JOURNAL OF CLINICAL MICROBIOLOGY, July 2008, p. 2189–2194 Vol. 46, No. 7
0095-1137/08/$08.00+0 doi:10.1128/JCM.00398-08

MDCK-SIAT1 Cells Show Improved Isolation Rates for Recent Human Influenza Viruses Compared to Conventional MDCK Cells▽†

Ding Yuan Oh,[1,2] Ian G. Barr,[1,2] Jenny A. Mosse,[2] and Karen L. Laurie[1]*

WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Road, Parkville, Melbourne, Australia 3052,[1] and School of Applied Sciences and Engineering, Monash University Gippsland Campus, Churchill, Victoria, Australia 3842[2]

Received 27 February 2008/Returned for modification 21 April 2008/Accepted 2 May 2008

The ability to isolate and propagate influenza virus is an essential tool for the yearly surveillance of circulating virus strains and to ensure accurate clinical diagnosis for appropriate treatment. The suitability of MDCK-SIAT1 cells, engineered to express increased levels of α-2,6-linked sialic acid receptors, as an alternative to conventional MDCK cells for isolation of circulating influenza virus was assessed. A greater number of influenza A (H1N1 and H3N2) and B viruses from stored human clinical specimens collected between 2005 and 2007 were isolated following inoculation in MDCK-SIAT1 cells than in MDCK cells. In addition, a higher titer of virus was recovered following culture in MDCK-SIAT1 cells. All A(H1N1) viruses recovered from MDCK-SIAT1 cells were able to agglutinate both turkey and guinea pig red blood cells (RBC), while half of the A(H3N2) viruses recovered after passage in MDCK-SIAT1 cells lost the ability to agglutinate turkey RBC. Importantly, the HA-1 domain of the hemagglutinin gene was genetically stable after passaging in MDCK-SIAT1 cells, a feature not always seen following MDCK cell or embryonated chicken egg passage of human influenza virus. These data indicate that the MDCK-SIAT1 cell line is superior to conventional MDCK cells for isolation of human influenza virus from clinical specimens and may be used routinely for the isolation and propagation of current human influenza viruses for surveillance, diagnostic, and research purposes.

The ability to isolate and propagate influenza virus is essential for the yearly surveillance of circulating virus strains and for further studies, such as antigenic and antiviral sensitivity analyses. Historically, embryonated chicken eggs have been used to propagate influenza viruses, and they are still used by most manufacturers to produce influenza vaccine today (2, 3, 4). Infection of immortalized mammalian cell lines in vitro with influenza virus has provided an alternative to egg inoculation (10, 13). Following the threat of an avian-derived H5N1 influenza pandemic, vaccine production via cell culture is currently being expanded, allowing more rapid production of influenza vaccine (8, 21, 29, 44).

Cell culture has been the preferred method for laboratory-based influenza virus isolation since the 1960s, as it is relatively simple, sensitive, and cost-effective. Many cell lines have been used, including BHK-21 (14), LLC-MK2 (38), SPJL (40), and Vero cells (11, 12). Madin-Darby canine kidney (MDCK) cells, however, have proved to be the easiest to handle, the most sensitive, and the most reliable cell line (10, 13, 27, 38) and remain the standard cell line for influenza virus propagation. Both human and avian influenza viruses can be isolated from MDCK cell culture with high viral titers (27, 42, 43), which may be attributed to the fact that both α-2,6- and α-2,3-linked sialic acid receptors are expressed on the surfaces of MDCK cells, and these are the primary receptors for human and avian influenza viruses, respectively (18, 35). However, unlike human respiratory cells, the level of α-2,6-linked sialic acid receptors on MDCK cells is relatively low, and thus, MDCK cells are not an ideal in vitro representation of the human respiratory system (1, 7, 19, 40). Matrosovich and colleagues established an MDCK cell line, MDCK-SIAT1, that was stably transfected with human CMP-*N*-acetylneuraminate:β-galactoside α-2,6-sialyltransferase, an enzyme that catalyzes the α-2,6-sialylation of galactose on glycoproteins or glycolipids. These MDCK-SIAT1 cells overexpress the α-2,6-linked sialic acid receptor compared to MDCK cells (25). Enhanced α-2,6-linked receptor levels should increase the number of interactions between human influenza virions and the cell surface, and hence, the avidity of the binding. This was indirectly demonstrated by MDCK-SIAT1 cells being more sensitive for assaying human influenza virus susceptibility to neuraminidase inhibitors than MDCK cells (25). Increased sensitivity to neuraminidase inhibitors was also seen in viruses passaged through another MDCK cell line transfected with the β-galactosidase α-2,6-sialyltransferase I gene (ST6Gal I) (16). In addition, in a small study with eight A(H3N2), seven A(H1N1), and five B viruses collected from 1999 to 2004, ST6Gal I cells showed increased isolation rates with A(H3N2) and B viruses, but not A(H1N1) viruses, as well as enhanced virus growth with all three types/subtypes if the samples had previously been grown in MDCK cells (16). However, neither of these studies addressed the potential for in vitro modifications to the virus during culture in cells overexpressing surface α-2,6-linked sialic acid receptors, a common feature seen in egg adaptation of viruses, where mutations of the hemagglutinin (HA) gene occur in

* Corresponding author. Mailing address: WHO Collaborating Centre for Reference and Research on Influenza, 45 Poplar Road, Parkville, Melbourne, Australia 3052. Phone: 61-3-9389-1340. Fax: 61-3-9389-1881. E-mail: karen.laurie@influenzacentre.org.

† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 14 May 2008.

response to the abundance of α-2,3-linked sialic acid moieties (18).

Possibly for this reason, or because of the lack of further reports on the usefulness of these cell lines for influenza virus isolation, few laboratories have adopted MDCK-SIAT1 or ST6Gal I cells for routine virus isolation or growth. In this study, we further assessed whether MDCK-SIAT1 cells were a viable alternative to conventional MDCK cells for isolation and propagation of recent influenza viruses. We found that MDCK-SIAT1 cells gave higher isolation rates and greater growth of influenza virus from human clinical specimens than MDCK cells. In addition, there was little change in the sequence of the HA gene on passaging in this modified MDCK cell line.

MATERIALS AND METHODS

Cells. MDCK cells (CCL-34; ATCC) and MDCK-SIAT1 cells (kindly provided by Hans-Dieter Klenk, University of Marburg, Marburg, Germany) were passaged in Dulbecco's modified Eagle's medium/HAM's F12 Coon's medium (SAFC Biosciences) supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 1× nonessential amino acids, 0.075% (vol/vol) sodium bicarbonate, 10 mM HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin (SAFC Biosciences), and 20 μg/ml amphotericin B (Fungizone; Bristol-Myers Squibb Company). The passage medium for MDCK-SIAT1 cells was further supplemented with 1 mg/ml G418 sulfate (Geneticin; Gibco). Viral infection studies were performed in the absence of FCS and G418. Cells were maintained at 37°C prior to infection and at 35°C following infection.

Clinical specimens and viruses. Influenza A(H1N1), A(H3N2), and B viruses were received from WHO National Influenza Centers, WHO Influenza Collaborating Centres, and other regional laboratories and hospitals in Australia, New Zealand, and the Asia/Pacific region. A full list of recovered viruses is provided in the supplemental material. Viruses were received as isolates passaged in cell culture or as original clinical samples (in which influenza A or B virus had been detected by immunofluorescence or by reverse transcription [RT]-PCR). Original clinical samples were collected as throat swabs, nasopharyngeal swabs, and throat washes. All clinical specimens and virus isolates were stored at −80°C prior to use, and clinical samples had been thawed at least once previously.

Virus infection and analyses. MDCK and MDCK-SIAT1 cells (5×10^5 per well) were seeded into 12-well plates (Cellstar; Greiner Bio-one) and allowed to grow to confluence overnight at 37°C in 5% CO_2. Monolayers were washed twice with Ca^{2+}/Mg^{2+}-free phosphate-buffered saline before incubation with 50 μl virus sample (clinical isolates were inoculated neat; cell- or egg-passaged virus was inoculated at 1/100 dilution) at 35°C and 5% CO_2 for 30 min. After inoculation, 3 ml medium (without FCS and G418) supplemented with 4 μg/ml trypsin was added to each well, and the cells were incubated at 35°C and 5% CO_2. The wells were monitored daily for virus growth by cytopathic effects, and after 4 days, the supernatant was collected and the presence of virus was assessed by hemagglutination using 1% turkey or guinea pig red blood cells (RBC). For serial passaging, cell supernatants were passaged another five times in the same cell line used for isolation before RNA extraction and sequencing. Viral titers in the supernatant were quantified by 50% tissue culture infectious dose ($TCID_{50}$) assay (41) with 10-fold dilutions of supernatant inoculated onto confluent monolayers in 96-well plates and allowed to grow for 4 days before being stained with 0.036% (wt/vol) neutral red. The virus was titered using MDCK cells for viruses that had been grown in MDCK cells and MDCK-SIAT1 cells for viruses that had been grown in MDCK-SIAT1 cells. All $TCID_{50}$ assays were performed in triplicate.

HA gene sequencing. For RT-PCR, viral RNA was extracted from 140 μl of infected cell culture supernatant or clinical sample using the QIAamp Viral RNA minikit (Qiagen). A 5-μl aliquot of RNA was used to amplify the HA-1 domain of influenza HA using gene-specific primers and SuperScript III One-Step RT-PCR with Platinum *Taq* (Invitrogen). Fragments amplified (and nucleotide positions) were as follows: H3 HA, 34 to 1101; H1 HA, 1 to 1317; and B HA, 20 to 1119 using primers H3 (forward, 5′-GACTATCATTGCTTTGAGCTAC, and reverse, 5′-CTATCATTCCCTCCCAACCAT), H1 (forward, 5′-AGCAAAAGCAGGGGAWAA, and reverse, 5′-ACAGCTGTGAATTGAGTGTT, or reverse, 5′-CCTCATAGTCGGCGAAATA), and B (forward, 5′-CTAATATCCACAAAATGAAGGC, and reverse, 5′-ACCAGCAATAGCTCCGAAGAAA). The RT-PCR consisted of 1 cycle of 50°C for 30 min and 94°C for 2 min and 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 1 min, followed by a final elongation step of 68°C for 7 min. The amplicons were visualized on a 2% (wt/vol) agarose gel. PCR products were purified for use in sequencing reactions using exonuclease I-shrimp alkaline phosphatase (ExoSAP-IT; USB Corporation). DNA sequencing was carried out using the above-mentioned primers in a 96-well plate format using an ABI Prism Dye Terminator III cycle-sequencing kit (Applied Biosystems), followed by the removal of excess dye terminators with a BigDye XTerminator purification kit (Applied Biosystems). The sequence was determined using an automated capillary DNA sequencer (ABI Prism 3700 at the Institute of Medical and Veterinary Science, Adelaide, Australia). Sequences were assembled using Lasergene Seqman package IV [DNAStar V7.2.1(1)].

Statistics. The data were analyzed using the MINITAB statistical analysis program at a confidence interval of 95%. Differences were considered statistically significant when the P value was <0.05. Median virus titers were compared using the nonparametric two-tailed Mann-Whitney U test; the number of virus isolates recovered in each cell line was analyzed using the chi-square test.

Nucleotide sequence accession numbers. The sequences of the HA-1 domain of the HA genes of all original clinical specimens analyzed by multiple passaging in the cell lines are available on GenBank/EMBL/DDBJ with accession numbers provided in the supplemental material.

RESULTS

The rate of isolation and titer of influenza virus from human clinical specimens are increased after growth in MDCK-SIAT1 cells compared to MDCK cells. To determine whether MDCK-SIAT1 cells had a higher rate of isolation of recent influenza viruses, MDCK-SIAT1 and MDCK cells were infected with a total of 125 stored clinical specimens (previously confirmed to be influenza virus positive) in duplicate collected during the period from 2003 to 2007 [39 A(H1N1), 53 A(H3N2), and 33 B]. Following 4 days in culture, the supernatants were analyzed for influenza virus by $TCID_{50}$ (performed in the same cell line used for isolation). Viruses were considered to have grown successfully if the $\log_{10}$ $TCID_{50}$/ml was ≥2. Of the stored clinical samples, 74% of A(H1N1), 91% of A(H3N2), and 39% of B viruses were successfully recovered in at least one of the cell lines (Fig. 1A). No viruses were isolated exclusively in MDCK cells. Two-thirds of the viruses grew in both cell lines, and the remaining viruses were recovered only in MDCK-SIAT1 cells (Fig. 1A). There was no correlation between the year of isolation and cell line recovery nor any correlation between virus origin and recovery patterns for A(H1N1) and B viruses (data not shown). For A(H3N2) viruses, a yearly breakdown revealed that the isolation pattern of the viruses appeared to be changing, particularly between 2006 and 2007, with a significant reduction in the ability of MDCK cells to isolate 2007 viruses compared to 2006 viruses ($P < 0.005$), unlike MDCK-SIAT1 cells, which were able to grow A(H3N2) viruses from both years (Fig. 1B). The median viral titers of clinical specimens were significantly higher in MDCK-SIAT1-grown influenza A cultures than in MDCK-grown cultures for A(H1N1) viruses ($P < 0.001$) and A(H3N2) viruses ($P < 0.0005$). No significant difference in the titer of B viruses was detected, but this may have been due to the small number of B viruses recovered (8 in MDCK cells and 13 in MDCK-SIAT1 cells) (Fig. 1C).

MDCK cell-passaged and egg-passaged influenza viruses propagate in MDCK-SIAT1 cells. To determine if MDCK-SIAT1 cells could support the growth of viruses previously cultured in MDCK cells or embryonated eggs, MDCK-SIAT1 cells (with MDCK cells as a control) were infected in duplicate with 10 MDCK cell-passaged viruses [3 A(H1N1), 4 A(H3N2), and 3 B/Malaysia/2506/2004-like] and 6 egg-passaged viruses [2 A(H1N1), 2 A(H3N2), and 2 B/Shanghai/361/2002-like] iso-

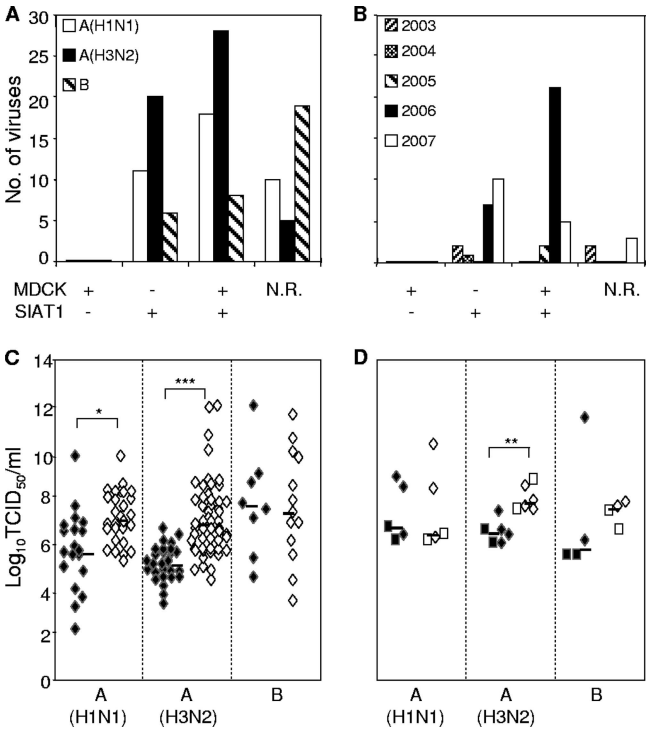

FIG. 1. Isolation and passaging of influenza virus samples in MDCK and MDCK-SIAT1 cells. Human clinical specimens and cell- and egg-passaged influenza virus samples were cultured in duplicate in MDCK or MDCK-SIAT1 cells for 4 days. The supernatants were collected, and influenza virus titers were measured by $TCID_{50}$ assay. (A) Total number of human clinical virus isolates recovered in each cell line. N.R., samples not recovered; SIAT1, MDCK-SIAT1 cells. (B) Recovery of A(H3N2) viruses from stored clinical samples by year of collection. (C) Titers for individual stored clinical samples (average of duplicates) isolated in either MDCK (black) or MDCK-SIAT1 (white) cells. (D) Titers for viruses previously isolated in eggs (squares) or MDCK cells (diamonds) and repassaged in MDCK (black) or MDCK-SIAT1 (white) cells. The medians are indicated by the horizontal bars. *, $P < 0.001$; **, $P < 0.005$; ***, $P < 0.0005$.

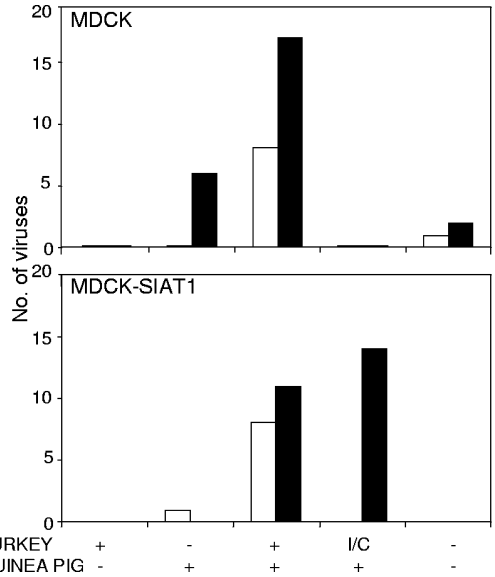

FIG. 2. RBC agglutination after cell culture. Human clinical specimens cultured in MDCK or MDCK-SIAT1 cells were assayed by hemagglutination assay with either guinea pig or turkey RBC. Shown are the numbers of hemagglutination-positive samples (titer > 1) for each RBC type from MDCK- or MDCK-SIAT1-cultured A(H1N1) (white) or A(H3N2) (black) viruses. I/C, incomplete agglutination (thus, the titer was not determined).

lated from 2003 to 2007. The supernatants were collected after 4 days and analyzed for virus by $TCID_{50}$ assay; 9/10 cell-passaged viruses were recovered in MDCK-SIAT1 cells and 10/10 in MDCK cells. One previously cell-passaged virus, B/Malaysia/174/2006, did not grow in MDCK-SIAT1 culture and grew in only one of two infected MDCK wells and thus was eliminated from any further analyses. All egg-isolated viruses grew in both cell lines. The median viral titers of cell- and egg-passaged viruses were significantly higher in MDCK-SIAT1-grown cultures than in MDCK-grown cultures for A(H3N2) viruses ($P < 0.005$), but no significant difference in growth of A(H1N1) or B viruses was detected (Fig. 1D).

RBC binding changes after passaging in cell lines. RBC agglutination is the method most commonly used to detect influenza virus particles in cell culture supernatants. To investigate changes to HA binding following cell culture of clinical specimens, randomly selected MDCK-SIAT1- and MDCK-passaged A(H1N1) ($n = 9$) and A(H3N2) ($n = 25$) viruses from 2005 to 2007 that grew in both cells lines were analyzed for the ability to agglutinate turkey and guinea pig RBC. Turkey (and chicken) RBC express high levels of α-2,3-linked sialic acid receptors and lower levels of α-2,6-linked sialic acid receptors than human and guinea pig RBC (19, 26). Following passage of A(H1N1) viruses in MDCK cells, the majority of viruses were able to agglutinate both turkey and guinea pig RBC (Fig. 2). One virus, A/Singapore/32/2006, was unable to agglutinate either type of RBC, possibly because the virus titer was very low (data not shown). Following passage of A(H1N1) viruses in MDCK-SIAT1 cells, the majority of samples agglutinated both turkey and guinea pig RBC (Fig. 2). One virus, A/Singapore/68/2005, was able to agglutinate only guinea pig RBC.

Agglutination patterns were different for A(H3N2) viruses after passage in the two cell lines. The majority of A(H3N2) virus samples passaged in MDCK cells were able to agglutinate both turkey and guinea pig RBC, while almost one-quarter of viruses could agglutinate only guinea pig RBC (Fig. 2). Following passage in MDCK-SIAT1 cells, all A(H3N2) viruses agglutinated guinea pig RBC. Interestingly, agglutination of turkey RBC by A(H3N2) viruses passaged in MDCK-SIAT1 cells was variable. Almost half of the viruses also agglutinated turkey RBC, and the remaining viruses had only partial agglutination with turkey RBCs (Fig. 2). This incomplete agglutination was characterized by difficulty in determining the titer of the virus. Repassage of "incomplete" samples isolated in MDCK-SIAT1 cells into MDCK cells restored the viruses' ability to agglutinate turkey RBC, while repassage of samples isolated in MDCK cells into MDCK-SIAT1 cells generally led to loss of the ability to agglutinate turkey RBC (data not shown). These data indicate that the incomplete agglutination patterns observed were a result of the cell passaging and not a property of the individual viruses.

The HA gene remains unchanged following viral passage in MDCK-SIAT1 cells. To determine if the passaging of influenza

TABLE 1. Sequence mutations in HA gene after passaging in MDCK or MDCK-SIAT1 cells[a]

HA-1 base pair mutation	HA-1 amino acid mutation	Cell passage result (no. of viruses with mutations/total no. of viruses sequenced)			
		MDCK		MDCK-SIAT1	
		P1	P5	P1	P5
A(H1N1)					
G606A	D190N		4/6		
C426G	N129K		1/6[b]		
A(H3N2)					
G316A	A106T	1/5	1/5		
T75A				1/5	1/5
B					
A590C	N197T	2/6	2/6	1/6	2/6
C596T	T199I		2/6		
C647T	S216F	3/6[b]	3/6[b]	3/6[b]	1/6[b]
G689A	G230D	2/6[b]	2/6	1/6	2/6
T45G		1/6	1/6	1/6	1/6
A594G		1/6	2/6	1/6	2/6
A816G		1/6	2/6	1/6	2/6
G843A		1/6	2/6	1/6	2/6
A906G			2/6	1/6	2/6

[a] Human clinical specimens were passaged five times in either MDCK or MDCK-SIAT1 cells in duplicate. The HA-1 domain of the HA gene was sequenced after passage 1 (P1) and passage 5 (P5), and mutations (silent and those resulting in an amino acid change) were recorded where there were changes from the sequence obtained directly from the clinical sample; amino acids are numbered in accordance with the H3 numbering system; base pairs are numbered with reference to actual sequence numbers of each subtype.

[b] Mixed population.

virus in MDCK-SIAT1 cells (or conventional MDCK cells) resulted in virus-cell adaptations, six A(H1N1), five A(H3N2), and six B influenza viruses that grew in both cell lines were passaged five times in each cell line. After each passage, the cytopathic effect was observed and RBC agglutination was measured to confirm virus growth. A(H3N2) samples cultured in MDCK cells agglutinated both turkey and guinea pig RBC after five passages, while A(H3N2) samples cultured in MDCK-SIAT1 cells were able to agglutinate only guinea pig RBC after one passage. Comparison of the HA-1 domains of the HA gene sequences of isolated viruses at passage 1 and passage 5 to those of viruses from original clinical specimens showed that the HA gene of A(H1N1) viruses did not mutate following a single passage in either cell line. However, two base pair changes were seen in A(H1N1) viruses following five passages in MDCK cells (Table 1). Replacement of G with A at 606 bp, leading to an amino acid change, D190N (H3 numbering system), was detected in four of six A(H1N1) MDCK-passaged viruses. A mixed population was detected in another sample, with replacement of C with G at 426 bp, producing an N129K mutation. No changes were seen in A(H1N1) viruses cultured multiple times in MDCK-SIAT1 cells compared to the sequence of the original clinical specimen. A synonymous change was detected in one A(H3N2) clinical specimen after passage in MDCK-SIAT1 cells (T replaced with A), and a single nonsynonymous change was detected in one A(H3N2) clinical specimen after passage in MDCK cells (G316A), leading to an alanine-to-tyrosine change at amino acid 106. These mutations appeared stable in the cell lines and were detected after both single and multiple passages (Table 1).

Interestingly, for influenza B viruses, eight base pair changes resulting in three amino acid changes were observed after initial passage in both MDCK cells and MDCK-SIAT1 cells (N197T, S216F, and G230D). Two of the nonsynonymous mutations were present as mixed populations in some of the cultures (S216F and G230D). Further mutations were detected after five passages in the two cell lines, with one nonsynonymous change in two of six MDCK-passaged viruses (T199I) and a synonymous change in both MDCK- and MDCK-SIAT1-passaged viruses (A906G). The HA sequence changes were seen for the same viruses in both cell lines, suggesting they represent a general adaptation to cell culture rather than a specific adaptation to culture in MDCK-SIAT1 cells.

DISCUSSION

In this study, the usefulness of MDCK-SIAT1 cells compared to conventional MDCK cells for isolation of influenza virus from stored human clinical samples was investigated. While previous small studies demonstrated some improvement in isolation rates, few laboratories routinely use MDCK-SIAT1 or the similar cell line ST6Gal I (16, 25). The data reported here confirmed that MDCK-SIAT1 cells could be used to isolate influenza A (H1N1 and H3N2) and B viruses more efficiently from stored clinical specimens than the parental MDCK cells in a large number of samples collected in 2006 to 2007. The viruses isolated had fewer amino acid mutations in the HA-1 domain of the HA gene following multiple passages in MDCK-SIAT1 cells than in MDCK cells. In addition, MDCK-SIAT1 cells were able to support higher levels of virus growth for A(H1N1) and A(H3N2) viruses than MDCK cells.

Infection of cells with influenza virus is limited by the number of virions in the clinical specimen and the avidity of the interaction between virions and receptors, which comprises both the individual affinity of the HA for sialic acid-linked receptors and the number of receptors available on the cell (or virion) surface. The increased level of α-2,6-linked sialic acid receptors on MDCK-SIAT1 cells should increase the avidity of the interaction and hence is likely to contribute to a higher recovery rate for human influenza viruses from stored clinical samples with MDCK-SIAT1 cells than with MDCK cells. No pattern was present between influenza A(H1N1) virus recovery and the year or location of a clinical specimen. However a yearly breakdown revealed that the receptor specificity of A(H3N2) viruses appeared to be drifting, with more viruses recovered in both cell lines in 2006 than in 2007, when the majority of A(H3N2) viruses were recovered only in MDCK-SIAT1 cells. There was no significant difference in the $\log_{10}$ $TCID_{50}$ titers of A(H3N2) samples from 2006 to 2007 recovered in MDCK-SIAT1 cells, suggesting that the amount of virus in the samples was not a limiting factor (data not shown). The density of HA on the virion surface may have altered between 2006 and 2007 A(H3N2) viruses, a feature seen with some H5N1 viruses (17). Most likely, however, the affinity of the HA for α-2,6-linked sialic acid receptors altered over time. Reduction in the affinity of A(H3N2) viruses has been reported previously, with a loss of binding to chicken RBC by A(H3N2) viruses seen over the past 10 to 15 years (15, 22, 26, 28). This may be attributed to a reduced affinity for sialic acid-linked

receptors, particularly α-2,6-linked receptors, which are at lower levels on chicken (and turkey) RBC than on guinea pig RBC (26). This antigenic drift appears to be more dramatic in the viruses assessed in this study, particularly between 2006 and 2007. Changes in HA sequence between A(H3N2) viruses from 2006 and 2007 viruses have been observed, particularly K140I, R142G, and N144D, which are in antigenic site 3 and lie close to the receptor binding region. Although a direct link between these specific mutations and receptor affinity has yet to be confirmed, these amino acids are found in a high proportion of A(H3N2) 2007 viruses but rarely in 2006 viruses, suggesting they may contribute to the observed changes in HA binding characteristics.

RBC agglutination of viruses isolated from cell or egg passage is typically used to assess culture-derived modifications and the specificity of viral HA for influenza virus receptors (18, 15, 22, 26, 28). In addition, HA inhibition assays with RBC are routinely used to monitor the antigenic drift of influenza viruses as part of the WHO Global Influenza Program (5). Therefore, any change in the ability of viruses to bind to RBC from different species following passage in MDCK and MDCK-SIAT1 cells is particularly important. Following passage in MDCK-SIAT1 cells, A(H3N2) viruses showed altered agglutination patterns, with the majority of A(H3N2) isolates unable to agglutinate turkey RBC completely. Changes have often been observed in RBC agglutination patterns following passage of viruses in embryonated chicken eggs compared to MDCK cells (18), but this is generally attributed to changes in HA specificity due to the increased proportion of α-2,3-linked receptors in eggs compared to MDCK cells and produces viruses equally capable of binding both chicken and turkey RBC (18). However there were few sequence changes in HA following passage in MDCK-SIAT1 cells compared to the original clinical sample and little variation in HA sequence between MDCK- and MDCK-SIAT1-passaged A(H3N2) viruses, despite the altered agglutination patterns. This further suggests that agglutination differences are a feature of passage in MDCK-SIAT1 cells. Differences in agglutination patterns have also been reported following passage of A(H3N2) viruses in Vero cells compared to MDCK cells (36), where viruses grown in Vero cells lost the ability to agglutinate chicken erythrocytes. While viruses isolated from both cell lines had identical HA-1 (and other gene) sequences, the HA from Vero cell-derived viruses had a different glycosylation pattern, with oligosaccharides of the high-mannose type, unlike MDCK-derived viruses. When some of these mannose residues were removed enzymatically, the Vero cell-derived viruses had the ability to agglutinate chicken erythrocytes restored. No such differences were observed with A(H1N1) viruses grown in Vero cells (36). As MDCK-SIAT1 cells were engineered by transfection with the human CMP-*N*-acetylneuraminate β-galactoside α-2,6-sialyltransferase gene, glycosylation patterns on the HA of budding viruses may also be altered. Thus, to ensure accurate detection of A(H3N2) viruses following passage in MDCK-SIAT1 cells, we advise the use of mammalian RBC for hemagglutination assays.

As shown here and previously reported, unlike embryonated chicken eggs, influenza virus propagation in MDCK cells results in little genetic variation in the HA-1 gene compared to the original infecting sample (6, 13, 20, 26, 30, 32, 33, 34, 37, 39). However, two interesting amino acid mutations were detected as a stable change in more than one virus. The D190N mutation has been previously been reported in egg-passaged A(H1N1) viruses (9, 31, 33, 24) and had been shown to dominate in egg-passaged A(H1N1) viruses even if it was originally present as a minor species (33). As the side chain at position 190 of HA interacts with the 9-hydroxyl group of sialic acid (45), the D190N mutation increases binding to chicken embryo chorioallantoic membranes and decreases the affinity of virus for α-2,6-linked sialic acid receptors (9). N197 (with T199) is an N-linked glycosylation sequon in the HA-1 region of recent influenza B viruses (N-X-T/S; amino acids 196 to 198 for B/Yamagata lineage and 197 to 199 for B/Victoria lineage). Although egg-passaged viruses predominantly select for variants that no longer have this glycosylation site, MDCK cells can support the growth of virus with or without glycosylation at position 197 (6, 37). Studies using cell- and egg-passaged viruses and viruses modified by reverse genetics that contain the mutation demonstrated that the binding affinity to horse RBC was inhibited by glycosylation; however, the loss of the glycosylation site did not affect the affinity for α-2,3- or α-2,6-linked sialic acid receptors. This suggests a role for steric hindrance at this amino acid site in the influenza B virus HA that may also affect the antigenicity of the virus (6, 37). Although we have sequenced only one sample from each passage in our studies, the use of high-fidelity *Taq* and the repeated incidence of the same mutation after multiple passages in MDCK cells confirm the importance of the D190N and N197T/T199I mutations. Analysis of HA sequences from viruses routinely passaged at the WHO Collaborating Centre in Melbourne in the past 3 years revealed that D190N occurred in 14% (9/64) of randomly selected MDCK-passaged viruses while the N197T/T199I mutations have also been detected in this and other laboratories (23).

This study showed that MDCK-SIAT1 cells, which expressed enhanced levels of α-2,6-linked sialic acid receptors, supported isolation of more recent human influenza viruses from a large panel of stored clinical samples and grew viruses to a higher titer than conventional MDCK cells. Most importantly, virus from stored clinical specimens that could not be recovered after inoculation in MDCK cells was recovered after inoculation in MDCK-SIAT1 cells. Thus, we propose that MDCK-SIAT1 cells or a similar line, such as ST6Gal-1 cells, be used routinely, along with or even in place of conventional MDCK cells, to isolate human influenza viruses to ensure an accurate representation of currently circulating influenza virus strains. This may be increasingly important if difficulty in isolating A(H3N2) viruses in MDCK cells, as seen in recent years, continues. Further studies will, however, be required to determine if MDCK-SIAT1 and other equivalent cell lines are as efficient as MDCK cells in isolating and propagating avian influenza viruses, such as A(H5N1) viruses from humans. While these cell lines have been shown to express slightly lower or similar levels of α-2,3-linked receptors (the receptor for avian influenza viruses) (16, 25), no comprehensive evaluation has been performed to date.

ACKNOWLEDGMENTS

We thank Hans-Dieter Klenk for providing MDCK-SIAT1 cells and the National Influenza Centres and other submitting laboratories for providing the human clinical specimens used for analysis in this paper. We also thank our colleagues at the WHO Influenza Collaborating Centre for expert technical assistance and advice.

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health and Ageing.

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