New technologies for the control of influenza

Koudstaal, W.

Citation for published version (APA):
Overexpression of \( \alpha-2,6 \)-Sialyltransferase in PER.C6® cells leads to increased susceptibility for human influenza strains


1Crucell Holland BV, Leiden, the Netherlands
2Health Protection Agency Centre for Infections, London, United Kingdom
3TNO Biosciences, Leiden, the Netherlands
4Department of Virology, Imperial College, St. Mary’s Campus, London, United Kingdom

Submitted for publication
Abstract

Annual update of the influenza vaccine relies on analysis of antigenic data generated using cultured clinical influenza isolates. However, the culture of infectious influenza virus from clinical specimens has become increasingly unreliable due to the low avidity for the sialic acid receptor of recent influenza A viruses. MDCK cells overexpressing $\alpha$-2,6-sialyltransferase have been shown to improve primary isolation and growth of these viruses compared to conventional MDCK cells. To test the generality of the approach we have generated a PER.C6 cell line stably overexpressing ST6Gal1, PER.C6-$\alpha$2,6ST and compared the susceptibility to infection and ability to support growth of recent human influenza strains with that of the parental cells. The data obtained demonstrate that increased expression of $SA\alpha2,6Gal$ did not produce increased viral titres. However, it did result in an increased recovery rate of influenza A viruses from limiting dilutions of virus inoculum. Furthermore, virus was isolated from a greater proportion of PCR positive clinical specimens in PER.C6-$\alpha$2,6ST cells compared to the unmodified cell line supporting the use of cell lines over-expressing $\alpha$-2,6-sialic acid for the isolation and recovery of human influenza viruses.

Introduction

Most influenza vaccines are currently produced in eggs. Efforts to develop cell culture systems as alternative production platforms have intensified in recent years due to the increasing demands for seasonal influenza vaccine and the need for faster methods of vaccine production, particularly in the light of the pandemic threat posed by highly pathogenic avian influenza viruses [1, 2]. For the surveillance of circulating influenza strains, embryonated chicken eggs were originally used, but since the 1960s, cell culture has been the preferred method for isolating influenza strains from clinical swabs. This is because cultivation of influenza viruses in eggs can lead to the selection of antigenic variants [3-7], and for some strains is impossible altogether. A recent example is the H3N2 influenza A/Fujian/411/02 virus that was predicted to be the dominant strain in the 2003-2004 season. Since the A/Fujian/411/02-like viruses did not replicate in eggs, it was difficult to produce a matched vaccine [8-10]. In fact, such a vaccine strain could not be produced in time for the seasonal vaccine production cycle. These problems might be overcome by using cell culture systems for vaccine production instead of eggs [11]. A number of cell types are currently being explored as substrate for influenza vaccine production including Madin-Darby canine kidney (MDCK) cells, African Monkey kidney (Vero) cells, and human retinal PER.C6 cells. Of these three, MDCK and Vero cells have additionally been assessed for their suitability for the isolation of influenza viruses [12-16]. Indeed, MDCK cells have become the standard for surveillance of circulating strains and clinical diagnosis [17]. Whether or not an influenza virus will grow on a cell line depends at least in part on the presence of the appropriate receptor. The haemagglutinin (HA) of influenza virus binds to oligosaccharides...
containing terminal sialic acid (N-acetyl neuraminic acid) linked to galactose on host cell surface glycoproteins or glycolipids (reviewed in [18]). The specificity of HA for its cellular receptor is a major determinant of host range. Avian strains preferentially bind to cells via sialic acid alpha-2,3 linked galactosyl sugar (SAα2,3Gal), whereas human strains bind to alpha-2,6 linked sialic acids (SAα2,6Gal) [19-22]. Both types of SA linkages are present on most cultured cell lines. In recent years, growth of clinically representative influenza isolates, particularly those of the H3N2 subtype has become increasingly difficult in MDCK cells due to changes in their receptor binding properties that have occurred during their continued circulation in the human host [23]. Overexpression of the human CMP-N-acetylneuraminic beta-galactosamide α-2,6-sialyltranferase in MDCK cells by transfection with the ST6Gal1 gene (also known as ST6Gal I and SIAT1) resulted in increased 2,6-sialylation of glycoconjugates on the cell surface [24, 25], which in turn resulted in improved isolation rates of recent human influenza viruses [17, 24, 25]. PER.C6 cells express both SAα2,6Gal and SAα2,3Gal on the surface [26], and faithfully replicate recent clinical isolates of influenza virus (Hartgroves et al. in press). However it has not been investigated whether the abundance of SA on PER.C6 cells limits the growth of recent influenza strains. To study this, we have generated a PER.C6 cell line stably overexpressing ST6Gal1, PER.C6-α2,6ST, and compared the susceptibility to infection and ability to support growth of recent human influenza strains with that of the parental cells. The data obtained demonstrate that increased expression of SAα2,6Gal did not produce increased viral titres. However, it did result in an increased recovery rate of influenza A viruses from limiting dilutions of virus inoculum. Furthermore, virus was isolated from a greater proportion of PCR positive clinical specimens in PER.C6-α2,6ST cells compared to the unmodified cell line supporting the use of cell lines over-expressing α-2,6-sialic acid for the isolation and recovery of human influenza viruses.

Materials and methods

Cells

PER.C6 cells were cultivated in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum and 0.01 M MgCl₂ at 37 °C in 10% CO₂ atmosphere. PER.C6-α2,6ST cells were cultivated in the same medium supplemented with Zeocin™ (Invitrogen). For infections, medium was replaced by AEM (Invitrogen) supplemented with 4 mM L-Glutamine (Invitrogen) and 3 µg/ml Trypsin/EDTA (Invitrogen). MDCK cells were obtained from the American Type Culture Collection and were maintained in the same conditions as above, except that no MgCl₂ and selection reagent were added to the medium.
Viruses

Human influenza A H1N1 and H3N2 viruses and influenza B viruses were isolated at the Health Protection Agency Centre for Infections, London UK. Clinical influenza isolates were grown on MDCK or MDCK-SIAT cells in serum-free Earl’s MEM (Invitrogen, Paisley) in the presence of 1 µg/ml TPCK-treated trypsin (Worthington, New Jersey). Influenza A and B virus reference strains were grown in 10 day embryonated eggs following standard protocols.

Construction of plasmid expressing the ST6Gal1 gene

The human α2,6-sialyltransferase gene, ST6 beta-galactosamide alpha-2,6-sialyltransferase 1, was cloned from a lung cDNA library using the DNA sequence provided in GenBank (Accession No. NM_003032), and a high-fidelity polymerase, Pfu (Stratagene). The full-length open reading frame of ST6Gal1 was amplified with primers 5’- CCAG GATCCGCCACCATGATTCACACCAACCTGAAG-3’ and 5’-TTTTTTTTCTTAAGTTAGCAGTGAATGGTCCGG-3’, containing an optimal Kozak sequence and BamHI restriction site, respectively. The PCR product was cloned into a pcDNA3-based plasmid with an enhanced CMV promoter (-735 to + 95, [27]). The resulting plasmid was called pcDNA2001-a2,6ST. The resulting plasmid was called pcDNA2001-a2,6ST. Next, an IRES and the zeocin resistance gene (Sh ble), from the SEAP-IRES-zeo construct [28] were cloned directly downstream of the ST6Gal1 gene, resulting in plasmid pcDNA2001-a2,6ST.IRES.zeo.

Establishment of a stable cell line expressing ST6Gal1

The pcDNA2001-a2,6ST.IRES.zeo plasmid was transfected into PER.C6 cells with Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Transfected cultures were cultivated in the presence of 0.6, 0.8, or 1.0 mg/ml Zeocin. After two weeks, individual clones surviving the selection were isolated and expanded from 96-well format to T80 culture flasks in serial passages. Cells were maintained in the presence of Zeocin except when they were used for viral infection.

Detection of SAα2,6Gal and SAα2,3Gal

The levels of α2,6- and α2,3-linked sialic acids on the cell surface were examined by a Fluorescence-activated cell sorter (FACS) analysis using linkage-specific lectins, as described previously [29]. In brief, cells were released from the solid support using TrypLE Select (Gibco), washed once with DMEM-10% FBS, and incubated in DMEM at 37 ºC for 1 hour to restore trypsin-digested receptors. Subsequently, approximately 0.5x10⁶ cells were washed with binding buffer (50 mM Tris-HCl, 0.15 M NaCl, 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂, 0.5% BSA, pH 7.5). After centrifugation, the cell pellet was incubated with 0.5 µg Digoxigenin (DIG)-labeled Maackia amurensis agglutinin, MAA (Roche) or 0.25 µg Biotinylated Sambucus nigra agglutinin, SNA (Vector Laboratories) in 50 µl binding buffer at 4 ºC for 1 hour. After centrifugation, the cell pellet was incubated with 0.5 µg Biotinylated Sambucus nigra agglutinin, SNA (Vector Laboratories) in 50 µl binding buffer at 4 ºC for 1 hour. After two washes with binding buffer, the cells were incubated with 2,5µl FITC-labeled anti-DIG Fab fragments (Roche) or 0.1 µl Streptavidin FITC conjugate (Caltag Laboratories) in 50 µl binding buffer at 4 ºC for 1 hour. After two more washes, cells were resuspended in CellFix (Becton Dickinson) and fluorescence intensities were quantified on a FACS Calibur flow cytometer (Becton Dickinson).
Northern Blotting (Hybridization)

Total RNA was isolated using TRIzol Reagent (Invitrogen), separated by denaturing formaldehyde/formamide gel electrophoresis and transferred to Hybond-N membrane (Amersham) according to the manufacturer’s protocol. DNA probes against the human ST6Gal1 and GAPDH genes were labelled with $^{32}$PdCTPs using random primers (octamers) (Rad Prime DNA Labeling system, Invitrogen).

α2,6ST activity assay

To measure the activity of CMP-N-acetylneuraminic beta-galactosamido-α,2,6-sialyltranferase in cell lysates, a solid-phase assay was used as described before [30]. Briefly, cells were washed twice with PBS, scraped in 0.25M sucrose, 20 mM Tris-HCl (pH 7.5) with 1:100 protease inhibitor mixture (Sigma) and lysed by three cycles of snap-freezing and thawing. Total protein content of each sample was determined by BCA protein assay (Pierce) and samples were stored at -80 ºC. A 96-well white Maxisorp plate (Nunc) was coated overnight at 4 ºC with 20 µg/ml Asialofetuin from fetal calf serum type I (Sigma) in bicarbonate buffer (0.015M Na$_2$CO$_3$, 0.035M NaHCO$_3$ pH 9.6), washed 3 times with PBS 0.05% Tween 20, blocked with 2% gelatin for 1 hour at RT, and washed again 3 times with PBS 0.05% Tween 20. Subsequently, 100 µg total protein of each cell lysate in 50 mM cacodylate buffer (pH 6.5) was mixed with 100 µM CMP-NeuAc (Sigma) and 10 mM MnCl$_2$ and incubated for 2 hours at 37ºC in the coated plate. As controls, cell lysates without CMP-NeuAc and 100 µU of Rat α2,6ST (Calbiochem) and Rat α2,3ST (Calbiochem) were included. Wells were washed 6 times with PBS 0.05% Tween 20 and incubated for 1 hour with 2 µg/ml SNA-Biotin (Vector Labs) in 100 µl PBS 0.05% Tween 20 and 2% BSA at RT. Next, binding of SNA-biotin was quantified using the Light Diagnostics ChemFLASH AquaLite® Streptavidin Conjugate Pack (Millipore) and a luminometer plate reader (Luminoskan Ascent, Thermo Labsystems).

Real-time RT-PCR

Multiplex real-time RT-PCR was performed as described previously on nucleic acid extracted directly from clinical samples using an automated method (Magnapure, Roche, UK)[31].

Haemagglutination assay

Haemagglutination assays were performed in v-bottomed microtitre plates using 50 µl of 0.5% suspensions of guinea pig red blood cells in PBS added to 50 µl virus serially diluted in PBS.

Plaque assay

Viral supernatants were titrated by plaque assay on MDCK cells in 6-well tissue culture plates. Viruses were diluted in appropriate buffer and incubated for 1 hour on cell monolayers. Following removal of the inoculum, cells were overlaid with serum-free DMEM/F12 (Invitrogen, Paisley) mixed with Agarose (MP Biomedicals) plus 1 µg/ml TPCK-treated trypsin (Worthington, New Jersey) and incubated for three days. Cells were fixed and stained with carbol
fuscin and plaques counted to determine viral titre (pfu/ml).

Statistical Analyses

Viral titres obtained on PER.C6 and PER.C6-α2,6ST cells (Figure 2) were log transformed and analyzed by one-way ANOVA, separately by subtype and by timepoint. The different strains per subtype have thus been analyzed together. Two alternative tests were applied. 1. A second ANOVA was run on the subset of strains for with at least one positive titer was observed. 2. A binomial test was applied on the non-zero status of the results. The alternative models were used to account possible non-normality of the data due to excess zeros.

The mean maximum dilution for each virus that results in viral replication in the limiting dilution experiment was compared using ANOVA with cell line as fixed factor and experiment as random factor (Figure 3).

Data describing isolation of virus from clinical swabs were analyzed using a generalized linear model with a binomial distribution using a Logit link function with cell line and virus subtype as factors and Ct value as continuous variable. Statistical analyses were performed using SPSS 15.0 (SPSS Inc., USA)

Results

Generation and characterization of PER.C6-α2,6ST

Near-confluent monolayers of PER.C6 cells were transfected with pcDNA2001-α2,6ST.IRES.zeo in which the ST6Gal1 gene is coupled to the Sh ble gene through an internal ribosome entry site (IRES). The zeocin resistance protein encoded by the Sh ble gene interacts stoichiometrically in a 1:1 ratio with zeocin and high Sh ble expressing clones can be selected by adding high concentrations of zeocin to the culture medium [28]. Such clones should also display the highest expression of ST6Gal1 due to the transcriptional link with the Sh ble gene. Selections were performed with a range of 0.6, 0.8, or 1.0 mg/ml zeocin. A total of 209 individual clones were isolated of which 112 could be expanded and screened for the abundance of α2,6- and α2,3-linked sialic acids on their surface using linkage-specific lectins.

Figure 1A shows the reactivity with S. nigra agglutinin, specific for α2,6-linked sialic acids, and M. amurensis agglutinin, specific for α2,3-linked sialic acids, of the PER.C6-α2,6ST clone 156 compared with the parental PER.C6 line. Clone 156 had the highest increase in reactivity with S. nigra agglutinin compared to parental PER.C6 cells (over 80% increase). In contrast, the reactivity with M. amurensis agglutinin decreased by 40% compared to PER.C6 cells.

Although clone 156 had been selected at the highest concentration of zeocin, no clear correlation was found between the zeocin concentration at which clones had been selected and binding of S. nigra agglutinin. We therefore compared the level of ST6Gal1 transcription in these cells with that in parental PER.C6 cells and also with that in PER.C6 cells transiently transfected with pcDNA2001-a2,6ST by northern blot.
In untransfected PER.C6 cells, no ST6Gal1 mRNA could be detected (Figure 1B, lane 3), whereas it is abundantly present in both PER.C6-α2,6ST cells and transiently transfected cells (Figure 1B, lanes 1 and 2). The increased size of the mRNA detected in the stable cell line reflects the presence of the IRES and zeocin resistance gene. A solid-phase assay for the activity of CMP-N-acetylneuraminic beta-galactosamide α-2,6-sialyltransferase, adapted from Mattox et al., [30], was used to assess if the increase in ST6Gal1 mRNA resulted in increased enzyme activity (Figure 1C). The α-2,6-sialyltransferase activity in PER.C6-α2,6ST cells was over 40 times that measured in PER.C6 cells. Despite the large increase in mRNA levels and enzyme activity, the abundance of α2,6-linked sialic acids on the cell surface increased less than twofold. This result is in line with what has been described for MDCK cells transfected with ST6Gal1 [24, 25] and likely reflects a limit of sialic acid that can be expressed on any cell.
Chapter 2

Analysis of influenza A virus growth in PER.C6-α2,6ST

Cell lines with increased cell surface expression of α2,6-linked sialic acids might be expected to replicate human influenza virus better than the parental cell line. The functional impact of increased expression of α2,6-linked sialic acids on the surface of PER.C6-α2,6ST cells was assessed. In parallel PER.C6 and PER.C6-α2,6ST cells were infected at an MOI of 0.01 with a panel of recent influenza A virus strains comprising six H3N2 and four H1N1 viruses that had been isolated from clinical samples collected in the 2006/07 season on MDCK or MDCK-SIAT cells. The H3N2 viruses were antigenically similar to either A/California/7/04 or A.Wisconsin/67/05 and the H1N1 viruses were A/New Caledonia/20/99-like. After 24, 48, and 72 hours, supernatants were harvested and viral titres were determined by plaque assay on MDCK cells. No statistically significant difference in titre obtained on PER.C6 or PER.C6-α2,6ST cells was found for either subtype at any timepoint (Figure 2). Since titres of viruses with a strong preference for α2,6-linked sialic acids may be underestimated when determined by plaque assay on MDCK cells, the supernatants of 48 and 72 hours post infection were also titrated on MDCK-SIAT cells.

**Figure 2.** Growth of a panel of influenza strains. PER.C6 and PER.C6-α2,6ST cells were infected with four recent clinical influenza H1, and six H3 strains with an MOI of 0.01. Twenty-four, 48 and 72 hours after infection, supernatants were harvested and viral titers were determined by plaque assay on MDCK cells. Closed circles: titers on PER.C6 cells, open circles: titers on PER.C6-α2,6ST cells. Bars indicate geomeans. The dotted lines indicate the limit of detection.
susceptibility of PER.C6 and PER.C6-α2,6ST cells for influenza MDCK-SIAT cells. Although average titres on MDCK-SIAT cells were 0.4 log (2.5x) higher than on MDCK cells (data not shown), this was the case for all viral supernatants whether harvested from PER.C6 and PER.C6-α2,6ST cells. Thus overexpression of SAα2,6Gal on the cell surface of PER.C6-α2,6ST did not result in higher viral titres following infection with laboratory passaged, recent human influenza A viruses.

**Virus recovery from limiting dilutions**

Increased expression of α2,6-linked sialic acid on the cell surface may enhance recovery of virus from low viral load inoculums such as would be present in original clinical material. To test this concept, serial dilutions of known virus composition were inoculated in parallel in PER.C6 and PER.C6-α2,6ST cells. The viruses used included two influenza A viruses A/Brisbane/10/07 (H3N2), and A/Eng/545/07 (H1N1) and three influenza B viruses B/Florida/4/06, B/Eng/552/07 (both B/Yamagata lineage) and B/Malaysia/2506/04 (B/Victoria lineage). Viral supernatants were harvested at 7 days post-infection and growth assessed by haemagglutination (HA) assay using guinea pig red blood cells. The susceptibility of both cell lines for each virus was determined by scoring the highest dilution of virus that was still capable of initiating a productive infection. The log difference between that titre established for PER.C6 or PER.C6-α2,6ST cells was calculated (Figure 3). Influenza A and B viruses were able to replicate in both PER.C6 and PER.C6-α2,6ST cells. For both subtypes of influenza A virus, propagation from low titre inoculum was more successful in PER.C6-α2,6ST cells. A 4-fold higher dilution of H3N2 virus and an 18-fold higher dilution of H1N1 virus scored positive for virus growth.

**Figure 3.** Susceptibility of PER.C6 and PER.C6-α2,6ST cells for influenza A and B virus. Serial dilutions of virus were inoculated into PER.C6 and PER.C6-α2,6ST cells. Viral supernatants were harvested seven days later and tested for the presence of virus by HA assay. The log difference in highest dilution that still resulted in the detection of each virus between both cell lines is plotted. Values greater than 0 indicate a higher susceptibility of PER.C6-α2,6ST cells. Dots represent means of 3 independent experiments. Bars indicate the 95% Confidence Interval (95%CI). The observed difference is statistically significant if the 95%CI does not cross the zero threshold.
in cells overexpressing α2,6-linked sialic acid. In contrast, influenza B viruses from either B/Victoria or B/Yamagata lineages were more readily propagated from low titre inoculum in unmodified PER.C6 cells. In particular, lineage influenza B viruses (B/Eng/552/2007 and B/Florida/4/2006) showed a statistically significant preference for PER.C6 cells. Higher dilutions of B/Eng/552/2007 (465-fold) and B/Florida/4/2006 (177-fold) yielded virus in PER.C6 cells compared to PER.C6-α2,6ST cells. Thus presence of additional α2,6-linked sialic acid did not improve infection rates by influenza B virus.

Isolation of human influenza viruses from RT-PCR positive clinical samples

To determine whether the difference in susceptibility of PER.C6-α2,6ST to infection by low titre inoculum virus was relevant in the context of primary virus isolation, 26 clinical samples identified as influenza virus positive by real-time RT-PCR (16 positive for H3, 5 positive for H1 and 5 positive for influenza B) were inoculated onto adherent PER.C6 and PER.C6-α2,6ST cells in parallel. Viral supernatants were harvested at seven days post-infection and virus titre determined by haemagglutination assay (Table 1). From the 26 RT-PCR positive specimens tested, five infectious viruses (3 H1N1, 1 H3N2 and 1 influenza B) were recovered after inoculation onto PER.C6-α2,6ST cells and two (both H1N1) were recovered on PER.C6 cells. The Ct-value in the real-time RT-PCR represents the number of PCR cycles required before the amount of cDNA crosses a detection threshold and is inversely proportional to the viral load in the clinical swab. In this semi-quantitative assay we found a negative correlation between the Ct-value and successful virus isolation (p=0.024). Furthermore, although not statistically significant (p=0.17), likely due to the small sample size, the applied statistical model suggests that virus was isolated from samples with higher Ct values on PER.C6-α2,6ST compared to PER.C6 cells (estimated difference of 5 PCR cycles). This result indicates that the former are more susceptible to infection.

Discussion

Annual update of the influenza vaccine relies on analysis of antigenic data (haemagglutination inhibition titres) generated using cultured clinical influenza isolates. Phenotypic testing of cultured virus is also useful for detection of antiviral resistance in circulating virus as occurred during the 2007-08 influenza season [32]. However, the culture of infectious influenza virus from clinical specimens is not always reliable. MDCK cells overexpressing α-2,6-sialyltransferase have been shown to improve primary isolation and growth of recent influenza A viruses [24, 25], and this can be explained by the rather low avidity of these viruses for the sialic acid receptor [23]. To test the generality of the approach of over-expression of sialic acid on cell lines to enhance growth of influenza viruses, we generated a modified PER.C6 cell line that displays 80% more human
Susceptibility of PER.C6 and PER.C6-α2,6ST cells for influenza

<table>
<thead>
<tr>
<th>Virus type</th>
<th>Number</th>
<th>Mean Ct-value (range)</th>
<th>Ct-value*</th>
<th>Virus titre (HAU)</th>
<th>Virus titre (HAU)</th>
<th>Strain name</th>
<th>Antigenicity*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PER.C6 cells</td>
<td>PER.C6-α2,6ST cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1N1</td>
<td>13</td>
<td>33.31 (28.43 - 37.67)</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31.99</td>
<td>40</td>
<td>40</td>
<td>A/England/660/07</td>
<td>A/Solomon Islands/3/06-like</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>25.53</td>
<td>&lt;5</td>
<td>80</td>
<td>A/England/12/08</td>
<td>A/Solomon Islands/3/06-like</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>23.77</td>
<td>20</td>
<td>20</td>
<td>A/England/40/08</td>
<td>A/Solomon Islands/3/06-like</td>
<td></td>
</tr>
<tr>
<td>H3N2</td>
<td>4</td>
<td>31.08 (28.02 - 33.31)</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>29.37</td>
<td>&lt;5</td>
<td>20</td>
<td>A/England/80200017/08</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>29.66 (20.09 - 34.67)</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td>&lt;5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>31.9</td>
<td>&lt;5</td>
<td>10</td>
<td>B/England/131/08</td>
<td>B/Florida/4/06-like</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1. Isolation of human influenza viruses from clinical samples in PER.C6 and PER.C6-α2,6ST cells**

- **Mean Ct-value (range)**: The average Ct-value with the range.
- **Ct-value**: The Ct-value for the virus titre.
- **Virus titre (HAU)**: The haemagglutination titre.
- **Strain name**: The strain name of the isolated virus.
- **Antigenicity**: The antigenicity of the isolated virus.

**Notes:**
- *Ct-value >38 = no influenza virus detected*
- **Antigenicity**: Derived from MDCK or MDCK-SIAT cultured viruses
- **Strain name and antigenicity** based on antigenic analysis with MDCK cells
- **Virus titre (HAU)**: Assayed in MDCK cells
- **NA**: Data not available

influenza receptor at the cell surface. This increase in the level of $\alpha$2,6-linked sialic acids on the surface of PER.C6-$\alpha$2,6ST cells with respect to that on the parental cells is modest compared to the difference in activity of the $\alpha$2,6Sialyltransferase (Figure 1C). Since the enzyme does not seem to be the limiting factor for further increase of the expression of $\alpha$2,6-linked sialic acids, it could be either its substrate, cytidine monophosphate-activated N-acetylneuraminic acid (CMP-NeuAc), or the acceptor oligosaccharide chains of glycoconjugates containing Gal($\beta$1,4)GlcNAc. Keppler et al., have shown that addition of CMP-NeuAc, or its precursors N-acetyl-D-mannosamine (ManNAc) and D-mannosamine (ManN) to the culture medium of hyposialylated subclones of a human B lymphoma cell line resulted in increased overall $\alpha$-2,6-sialylation of cell surface glycoconjugates [33]. Culturing PER.C6 cells and PER.C6-$\alpha$2,6ST cells in the presence of a dose range of these substrates did not result in increased sialylation (data not shown). We therefore hypothesize that the availability of target N-acetyllactosamine moieties on glycoproteins and glycolipids limits the increase of $\alpha$2,6-linked sialic acids on PER.C6-$\alpha$2,6ST cells. This is consistent with the observation that the increase in SNA binding to PER.C6-$\alpha$2,6ST cells is accompanied by a decrease in MAA binding, which has also been seen in MDCK-SIAT1 cells and has been proposed to be due to competition between ST6Gal1 and $\alpha$2,3sialyltransferases that also target Gal($\beta$1,4)GlcNAc, like ST3Gal III and ST3Gal IV [24].

We compared isolation of human influenza viruses from clinical material on PER.C6 and PER.C6-$\alpha$2,6ST cells during a short period of the winter season 2007-08 (Table 1). The predominant virus circulating during this period in the UK was A/H1N1 subtype although influenza A/H3N2 and influenza B viruses were sporadically identified. A small panel of 26 clinical specimens, identified by real-time RT-PCR as positive for influenza A or B viral RNA, were randomly selected for testing from samples received in the Respiratory Virus Unit at HPA Centre for Infections, London. From 5 of these, viruses were recovered on PER.C6-$\alpha$2,6ST cells, including representatives of all human influenza subtypes. Only a subset of those isolates grew on PER.C6 cells suggesting that the increased display of $\alpha$-2,6-linked sialic acid on the cell surface enhanced the chance of virus isolation, in line with what has been found for MDCK cells overexpressing ST6Gal1 [17, 25]. The success of the PER.C6-$\alpha$2,6ST cell line in recovering influenza A virus from clinical material is consistent with the data in figure 3 that show virus can be propagated from lower titre inocula in this modified cell line than when the unmodified PER.C6 cells are used. It can be envisaged that the threshold for infection of PER.C6-$\alpha$2,6ST cells by influenza A viruses may be lower than PER.C6 cells due to the abundance of the appropriate receptor on the cell surface. In clinical material, where viral load is a limiting factor, use of a cell line with abundant $\alpha$2,6-linked sialic acid on the cell surface would be an advantage for isolation of human influenza A viruses.

In contrast to influenza A, influenza B viruses of both B/Yamagata and B/Victoria lineages that had been amplified in eggs showed a growth advantage in PER.C6 cells compared
Susceptibility of PER.C6 and PER.C6-α2,6ST cells for influenza to PER.C6-α2,6ST cells (Figure 3). Compared to influenza A, less is known about influenza B virus receptor binding preferences. The structure of influenza B HA complexed with avian or human receptor analogs indicates that B HA interacts with both receptors but forms more extensive interactions with both sialic acid and the asialo chain of the avian receptor compared to only limited interactions with the sialic acid portion of the human receptor [34, 35]. The presence of glycosylation at position 196-198 in the vicinity of the B HA receptor binding pocket may sterically hinder the HA interactions with the avian receptor chain [35, 36]. Indeed, growth of influenza B virus in eggs, which are rich in the avian receptor, is known to generate egg-adapted mutants that have lost this potential glycosylation site [6] and these viruses display a concurrent change in antigenicity and receptor binding properties [7, 36-39]. In our studies the egg-grown influenza B viruses B/Eng/552/07; B/Florida/4/06 and B/Mal/2506/04 lack glycosylation at 196-198 and are expected to form high avidity interactions with the avian receptor which is more abundant on the surface of the unmodified cells and this accounts for the improved virus recovery observed. Interestingly, the clinical isolate B/England/131/2008, which retains the potential glycosylation site at 196-198, was isolated on PER.C6-α2,6ST only. This suggests that clinical samples of influenza B viruses that are not egg-adapted may, like influenza A viruses, be more readily isolated on cell lines modified to overexpress the human receptor. It will be interesting to test this in the future during influenza seasons when the frequency of influenza B virus infections is higher.

The clinical specimens used in this study included nose or throat swabs from different sources and geographic locations in the UK. Nineteen of the 26 specimens had a Ct-value greater than 30 in a real-time RT-PCR assay, which is indicative of a low viral load. Next to factors that will influence viral load, such as timing of specimen collection after onset of illness and specimen quality, the proportion of viable virus in the sample and subsequent success of virus isolation in any cell line will also be dependant on factors such as temperature during storage and postal transfer to the laboratory. An optimized haemagglutination assay protocol using guinea pig red blood cells was used for virus detection in PER.C6 and PER.C6-α2,6ST cells, which although relatively insensitive compared to other methods such as quantitative PCR, allowed fast detection and an indirect comparison to be made with the results obtained with the routine virus isolation procedure using MDCK and MDCK-SIAT cells at the HPA Centre for Infections. From the 26 PCR positive samples used in this study, 8 and 9 isolates had been recovered on MDCK and MDCK-SIAT cells, respectively. Interestingly, the H3N2 virus A/England/802000017/2008 that was isolated on PER.C6-α2,6ST cells did not grow on conventional MDCK or MDCK-SIAT cells, suggesting that the modified PER.C6 cells could be useful for growing viruses that are difficult to propagate in other cell lines. Since such viruses may have diverse antigenic or receptor binding properties, isolation of human viruses in novel cell lines such as the PER.C6-α2,6ST cell described here could be an important adjunct for surveillance of changing properties of influenza viruses.
Acknowledgements

We thank Professor Maria Zambon for scientific input and critical review of the manuscript, staff at the Respiratory Virus Unit, HPA Centre for Infections, London for technical assistance and Martin Koldijk and Jaco Klap for statistical analyses.

PER.C6® is a registered trademark owned by Crucell Holland B.V.

References

5. Katz JM, Naeve CW and Webster RG. Host cell-mediated variation in H3N2 influenza viruses. Virology 1987;156:386-95


17. Oh DY, Barr IG, Mosse JA and Laurie KL. MDCK SIAT-1 cells show improved isolation rates for recent human influenza viruses compared to conventional MDCK cells. J Clin Microbiol 2008


23. Asaoka N, Tanaka Y, Sakai T, Fujii Y, Ohuchi R and Ohuchi M. Low growth ability of recent influenza clinical isolates in MDCK cells is due to their low receptor binding affinities. Microbes Infect 2006;8:511-9


