Vaccine 37 (2019) 4543–4550

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Haemagglutinin stability was not the primary cause of the reduced effectiveness of live attenuated influenza vaccine against A/H1N1pdm09 viruses in the 2013–2014 and 2015–2016 seasons

Lauren Parker *, Lydia Ritter, Wen Wu [1], Ruben Maeso [2], Helen Bright, Oliver Dibben

Flu-BPD, AstraZeneca, Liverpool, UK

ARTICLE INFO

Article history:
Received 12 February 2019
Received in revised form 7 June 2019
Accepted 10 June 2019
Available online 3 July 2019

Keywords:
Influenza
Live attenuated influenza vaccine
Thermostability
pH stability
Haemagglutinin
2009 pandemic A/H1N1 (A/H1N1pdm09)
Vaccine effectiveness

ABSTRACT

During the 2013–2014 influenza season, the quadrivalent live attenuated influenza vaccine (QLAIV), had lower than expected vaccine effectiveness (VE) against circulating A/H1N1pdm09 viruses in the USA. The underlying reason proposed for this was that the A/H1N1pdm09 vaccine strain, A/California/07/2009 (A/CA09), had a thermally unstable haemagglutinin (HA) protein. Consequently, a new A/H1N1pdm09 candidate strain, A/Bolivia/559/2013 (A/BOL13), was developed for inclusion in the 2015–2016 QLAIV. A key parameter for selection of A/BOL13 was its more thermostable HA phenotype compared with A/CA09. During the 2015–2016 season, QLAIV containing A/BOL13 was found in some studies to have improved, but still with suboptimal, VE against circulating A/H1N1pdm09 viruses and was not recommended for use by the CDC in the US market in the 2016–2017 influenza season. This suggested that improved HA thermostability had not entirely resolved the reduced VE observed. One hypothesis for this was that, by improving thermostability, the A/BOL13 HA protein had been over-stabilised, compromising its activation at the low endosomal pH required for successful viral entry. Here we demonstrate that, while the A/BOL13 HA protein is more stable than that of A/CA09, its thermal and pH stability were comparable with historically efficacious LAIV strains, suggesting that the HA had not been over-stabilised. Furthermore, studies simulating potential heat exposure during distribution by exposing QLAIV nasal sprayers to 33 °C for 4 h showed that, while remaining within product specification, A/CA09 viral potency was statistically decreased after 12 weeks at 2–8 °C. These data suggest that although unfavourable HA protein stability may have contributed to the reduced VE of A/CA09 in 2013–2014, it was unlikely to have affected A/BOL13 in 2015–2016. We conclude that HA stability was not the primary cause of the reduced effectiveness of LAIV against A/H1N1pdm09 viruses in the 2013–2014 and 2015–2016 seasons.

1. Introduction

Influenza is a significant cause of global morbidity and mortality, which causes annual seasonal global epidemics, and pandemic outbreaks at irregular intervals. The most effective means of preventing and controlling influenza infection is vaccination [1–3]. There are currently three main types of vaccine licensed and available for commercial use; inactivated, recombinant, and live attenuated influenza vaccines (LAIV). Trivalent LAIV containing representative A/H1N1, A/H3N2, and B-Victoria or B-Yamagata lineage strains, was licensed for use in the USA in 2003 and in the EU in 2011 [4,5]; quadrivalent LAIV (QLAIV) containing representative pandemic 2009 H1N1 (A/H1N1pdm09), A/H3N2, and both B-virus lineages, was licensed and approved for use in the USA in 2012 and in the EU in 2013 [6,7].

During the 2013–2014 influenza season, suboptimal vaccine effectiveness (VE) was observed for the A/H1N1pdm09 LAIV component, A/California/07/2009 (A/CA09), against circulating viruses of this subtype [8]. Previously, it was proposed that thermal instability of the A/CA09 haemagglutinin (HA) protein may have contributed to its reduced VE in 2013–2014 [8]. A key amino acid residue in the HA stalk region, E374, was identified as playing a significant role in the stability of A/H1N1pdm09 viruses isolated in the initial stages of the 2009 pandemic [9,10]. From 2010, viruses of this subtype naturally evolved the E374K substitution [11] which resulted in improved temperature and pH stability [9]. Increased outdoor temperature during LAIV lot unloading at US

* Corresponding author at: AstraZeneca UK, Unit 6 Renaissance Way, Speke, Liverpool L24 9JW, UK.
E-mail address: parkerla@medimmune.com (L. Parker).
[1] Current address: Cambridge Data Scientific, Barnet, UK.
[2] Current address: Seqirus Vaccines Ltd, Liverpool, UK.

https://doi.org/10.1016/j.vaccine.2019.06.016
0264-410X/

distributors was shown to statistically correlate with reduced LAIV VE against A/H1N1pdm09 viruses [8]. Incidentally, A/CA09 also proved to be problematic for inactivated influenza vaccine (IIV) manufacturers as a candidate vaccine virus (CVV) and antigen product. The CVV propagated inefficiently in embryonated eggs, gave low HA antigen yields, and had a reduced shelf-life due to its predisposition to HA degradation [12–14].

To replace A/CA09, an alternative A/H1N1pdm09 strain, A/Bolivia/559/2013 (A/BOL13), was identified as a suitable CVV with a more thermostable HA phenotype and was subsequently included in the 2015–2016 QLAIV. However, A/BOL13 also resulted in reduced VE in the US and, in 2016, the Advisory Committee on Immunization Practices (ACIP) withdrew their recommendation for the use of QLAIV in the 2016–2017 influenza season. During the same 2015–2016 season, studies conducted in the UK and Finland reported moderate, but suboptimal VE for QLAIV against circulating A/H1N1pdm09 viruses [15].

Several hypotheses have been proposed to explain the observed suboptimal VE of the QLAIV A/H1N1pdm09 component in the USA in recent years, including reduced viral fitness, viral interference in QLAIV, presence of an abundance of defective-interfering genomes, and previous vaccination history of recipients [16–20]. Another hypothesis is that by improving the thermostability of the A/BOL13 HA protein, it had become over-stabilised in the context of acid-dependent membrane fusion [21], reducing its ability to efficiently complete the viral replication cycle. Exposure of the viral HA to increased temperature and pH can cause an irreversible conformational change, resulting in the exposure of the fusion peptide, and subsequent activation of the HA protein [22–24]. While this is an essential process in the influenza virus life cycle, viruses with high pH and temperature sensitivity have the potential to become inactivated when exposed to environmental conditions, rendering them unable to infect cells due to premature activation of the viral HA.

While determinants of A/H1N1pdm09 HA protein stability have been described to an extent, the HA stability profile of clinically effective LAIV viruses is not yet understood. Here, we assessed the phenotypes of both A/CA09 and A/BOL13, along with a panel of historic, clinically effective LAIV strains to determine the roles of HA protein thermal and pH stability in the reduced VE of A/H1N1pdm09 LAIV viruses. Historical LAIV strains included in this study were A/New Caledonia/20/1999 (A/NC99), A/Sydney/5/1997 (A/SYD97) and B/Massachusetts/02/2012 (B/MASS12); representative of clinically effective, pre-pandemic A/H1N1, A/H3N2 and B-Yamagata subtypes, respectively [25–32].

In these studies, we confirm that the A/CA09 LAIV virus is more temperature and pH unstable than historical LAIV influenza A viruses with proven VE. These remain a possible contributing factor to A/CA09 reduced VE against circulating A/H1N1pdm09 strains. We also demonstrate that the A/BOL13 HA protein has a comparable pH and thermostability profile to historically efficacious influenza A LAIV strains. As suboptimal VE was still observed for A/BOL13, despite its improved pH and thermostability profile, we suggest that while an important consideration in CVV selection, HA stability was not the primary cause of the reduced effectiveness of LAIV against A/H1N1pdm09 viruses in the 2013–2014 and 2015–2016 influenza seasons.

2. Materials and methods

2.1. Cells and viruses

293 T cells and A549 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) (Gibco™; Thermo Fisher Scientific; Cat. No. 11960044) containing 10% heat-inactivated foetal bovine serum (v/v) (FBS) (Gibco™; Thermo Fisher Scientific; Cat. No. 10500056), 1% penicillin-streptomycin (v/v) (Gibco™; Thermo Fisher Scientific; Cat. No. 15140122), and 1% 200 mM L-Glutamine (v/v) (Gibco™; Thermo Fisher Scientific; Cat. No. 25030018).

Recombinant viruses used in this study were generated using an 8-plasmid reverse genetics system as previously described [33,34] and propagated in the allantoic cavity of 10- to 11-day-old embryonated hens' eggs. These viruses were LAIV 6:2 reassortants carrying the HA and neuraminidase (NA) gene segments of A/CA09, A/BOL13, A/NC99, A/SYD97, or B/MASS12, and the six internal gene segments (PB2, PB1, PA, NP, M, and NS) of cold-adapted A/Ann Arbor/6/1960 [35] or cold-adapted B/Ann Arbor/1/1966 [36].

Viruses were titrated by focus-forming assay (FFA) as previously described [37] and all were obtained from the Influenza Manufacturing Science and Technologies division of AstraZeneca UK Ltd, Liverpool.

2.2. Thermostability assays

2.2.1. Accelerated thermostability

The thermostability of the HA protein was determined by measuring the loss of HA titre after incubating viruses at 47.5 °C, 50 °C, 52.5 °C, 55 °C, 57.5 °C, 60 °C, 62.5 °C, and 65 °C for 20 min, or after an incubation hold at 57.5 °C for 60 min. Viruses were diluted to a standard HA titre of 512 HA units prior to incubation. All HA assays were performed using 0.5% chicken red blood cells (Envigo, Netherlands).

2.2.2. Long-term thermostability of fluenz® nasal sprayers

To determine if there was a significant virus potency loss after exposure to a high temperature, QLAIV nasal sprayers from the 2013–2014 season, containing A/CA09, A/Texas/50/2012 (A/TEX12), B/Brisbane/60/2008 (B/BRIS08), and B/MASS12 formulations, were incubated at 33 °C for 4 h, and then held at 4 °C for 12 weeks. A control group, not exposed to 33 °C, was included. Every 7 days during the 12-week period, samples were taken from each group, and all four viruses present in the sprayer were titrated by FFA assay as previously described [37].

To determine if the virus potency degradation slopes were significantly different between the control and 33 °C groups for each virus, the following statistical model was used $y_{ijkl} = \alpha_0 + \alpha_i + \beta_0 x_k + \beta_i x_k + a_{j(i)} + b_{j(i)} x_k + \varepsilon_{ijkl}$ [38]. Full statistical methodology is detailed in the supplementary information.

2.3. pH inactivation assay

The pH value at which there was a 99% reduction in infection was determined by inoculating A549 cells with viruses that had been pre-treated with Dulbecco's phosphate-buffered saline (DPBS) (Gibco™; Thermo Fisher Scientific; Cat. No. 14040117) adjusted to pH 7, 6, 5.8, 5.6, 5.4, 5.2, or 5, with 0.1 M citric acid. LAIV viruses were diluted to a multiplicity of infection (MOI) of 10 in each buffer and incubated at room temperature for 30 min. After incubation, each virus-pH buffer was neutralised by addition of DMEM containing 1% penicillin-streptomycin and 1% 200 mM L-Glutamine and inoculated onto A549 cells. Cells were incubated at 33 °C, 5% CO_2 for 18 h before fixing, permeabilising, and staining for viral nucleoprotein (NP) protein (anti-influenza A NP, Bio-Rad Cat. No. MCA400 and anti-Influenza B NP, Bio-Rad Cat. No. MCA403). In addition, cells were counter-stained with Hoechst (Invitrogen™; Thermo Fisher Scientific; Cat. No. H21491).

Stained cells were visualised with a 4x objective using an ImageXpress Micro 4 (Molecular Devices, LLC, Sunnyvale, CA, USA). Hoechst and NP staining were visualised using DAPI and FITC

filters, respectively. The 99% reduction in infection for each pH-treated virus sample was calculated as a percentage of total infected cells at pH 7 using the MetaXpress analysis software (MetaXpress; Molecular Devices, LLC, Sunnyvale, CA, USA). HA activation pH values for each virus were determined by interpolation of a sigmoidal standard curve using a four-parameter logistical equation (GraphPad Prism 7 software). The loss of infectivity due to low-pH treatment is a result of the viral HA protein being irreversibly activated prior to inoculation onto the A549 cells.

2.4. Fusion assay

Cell-to-cell membrane fusion mediated by the viral HA protein was examined in 293 T cells transiently expressing green fluorescent protein (GFP). GFP was cloned into the vector pVitro-neo-mcs (Invivogen, San Diego, CA, USA) and transfected into 293 T cells in Collagen I-coated 24-well plates (Gibco™; Thermo Fisher Scientific; Cat. No. A1142802) using Lipofectamine® 3000 (Invitrogen™; Thermo Fisher Scientific; Cat. No. L3000015) as per the manufacturer's recommendations. Six hours post-transfection, cells were infected with virus diluted to a MOI of 10 in Opti-MEM (Gibco™; Fisher Scientific; Cat. No. 31985047) containing 1% penicillin–streptomycin, and incubated at 33 °C, 5% CO_2 for 18 h. After the incubation period, cells were washed once with DPBS, followed by a 5-minute treatment with a trypsin-like enzyme (TrypLE) (Gibco™; Thermo Fisher Scientific; Cat. No. A121177-01) diluted 1:50 in DPBS, to cleave the cell surface-expressed HA0 protein into HA1 and HA2 subunits. The TrypLE was inactivated by addition of DPBS containing 10% FBS, which was then removed and replaced with DPBS adjusted to pH 7, 6, 5.8, 5.6, 5.4, 5.2, or 5 with 0.1 M citric acid. Cells were incubated with pH buffer at 33 °C for 15 min and neutralised by addition of DMEM containing 10% FBS before incubating at 33 °C, 5% CO_2 for 2 h. Cells were then imaged for GFP fluorescence of fused cells under 10x magnification using an Eclipse Ti-S microscope (Nikon, Tokyo, Japan).

3. Results

3.1. A/H1N1pdm09 A/CA09 is less thermostable when compared with historical LAIV viruses with proven VE

Accelerated thermostability assays were performed on LAIV viruses A/NC99, A/CA09, A/SYD97, and B/MASS12 to determine the cut-off temperatures at which the ability of each virus to haemagglutinate chicken red blood cells was lost. Viruses were incubated for 20 min at temperatures ranging from 47.5 °C to 65 °C (Fig. 1a) or at 57.5 °C with samples taken at a range of time points over a 60-minute period (Fig. 1b). Both assay formats showed that A/CA09 loses its ability to agglutinate chicken red blood cells at a temperature of 57.5 °C. These results corroborate those previously published, describing the poor HA stability of the LAIV A/CA09 and wild-type A/H1N1pdm09 viruses isolated in the initial stages of the 2009 pandemic [9,10,13]. LAIV A/NC99, A/SYD97, and B/MASS12 viruses demonstrated greater HA stability, all only suffering a reduction in HA titre after 20 min of incubation at 65 °C (Fig. 1a) and remaining thermostable throughout a 60-minute incubation at 57.5° (Fig. 1b).

3.2. A/H1N1pdm09 strain A/BOL13 has improved thermostability, and is comparable with pre-pandemic A/NC99

As it was deemed likely that the heat labile HA of A/CA09 was the cause of its reduced VE [8], an alternative LAIV A/H1N1pdm09 strain, A/BOL13, was developed for inclusion in the 2015–2016 QLAIV. One of the key attributes required of the new strain, in addition to its antigenic similarity to circulating A/H1N1pdm09 viruses, was that it should have a more thermostable HA when compared with its predecessor. A/BOL13 demonstrated improved stability when compared with A/CA09 in accelerated thermostability assays (Fig. 2a and b) and was comparable with A/NC99, a pre-pandemic H1N1 strain with proven VE. A/BOL13 remained able to agglutinate chicken red blood cells up to a cut-off temperature of 62.5 °C (Fig. 2a) and was stable over a 60-minute incubation at 57.5 °C (Fig. 2b).

3.3. A/H1N1pdm09 A/CA09 is less pH stable when compared with A/H1N1pdm09 A/BOL13

Despite its thermostable HA profile, inclusion of A/BOL13 in the 2015–2016 LAIV did not entirely resolve the suboptimal VE observed for A/CA09. One explanation for this was that, by improving its thermostability, the HA of A/BOL13 had been over-stabilised, and as a result did not undergo efficient virus-endosome membrane fusion at low-endosomal pH. To test this, a quantitative pH inactivation assay (Fig. 3a) was performed on the same panel of viruses.

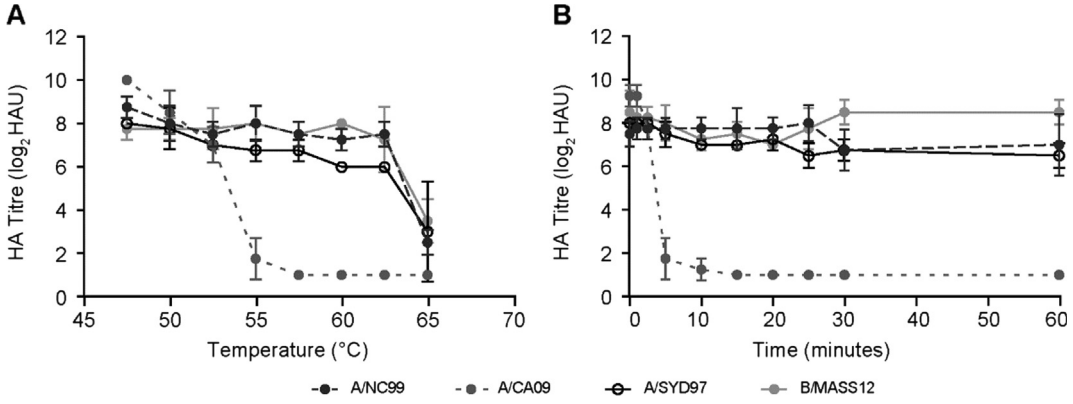

Fig. 1. Accelerated thermostability of A/CA09 A/H1N1pdm09 virus compared with historical live attenuated influenza vaccine viruses with proven efficacy. A/NC99, A/CA09, A/SYD97, and B/MASS12 viruses were incubated at temperatures ranging from 47.5 °C to 65 °C for 20 min (A) or were held at 57.5 °C and sampled over a 1-hour period (B). Haemagglutinin assays were performed on all samples after incubation using 0.5% chicken red blood cells to measure haemagglutinin titre (log_2HAU, y axis) after heat exposure.

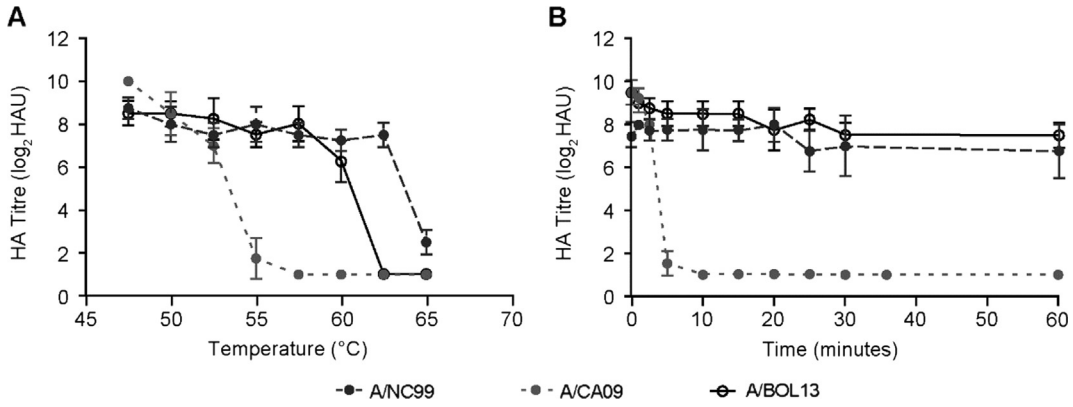

Fig. 2. Accelerated thermostability of A/BOL13 A/H1N1pdm09 virus compared with A/CA09 and A/NC99 viruses. A/NC99, A/CA09, and A/BOL13 viruses were incubated at temperatures ranging from 47.5 °C to 65 °C for 20 min (A) or were held at 57.5 °C and sampled over a 1-hour period (B). Haemagglutinin assays were performed on all samples after incubation using 0.5% chicken red blood cells to measure haemagglutinin titre (log_2HAU, y axis) after heat exposure.

A/NC99, A/CA09, A/BOL13, A/SYD97, and B/MASS12 viruses were treated with a range of pH-adjuster buffers prior to infection of A549 cells. Cells were stained for viral NP and DAPI after 18 h of infection, and infected cells were quantified on a high content imager in order to calculate percentages of infected and uninfected cells. The loss of infectivity due to low-pH treatment is a result of the viral HA protein being irreversibly activated prior to inoculation of the A549 cells.

Activation pH was approximately 5.1 for A/NC99, A/BOL13, and A/SYD97, and approximately 5.4 for A/CA09 and B/MASS12 (Fig. 3a). These data agree with those previously reported for LAIV and wild-type A/CA09 and recent A/H1N1pdm09 wild-type viruses possessing the K374 (HA2) amino acid, in which the fusion pH was reduced from 5.4 to 5.0 in viruses carrying this residue [9].

A qualitative GFP cell-to-cell fusion assay was then employed to confirm these data (Fig. 3b). Briefly, A/NC99, A/CA09, A/BOL13, A/SYD97, and B/MASS12 viruses were used to infect 293T cells transiently expressing GFP. Infected cells were treated with a trypsin-like enzyme and pH-adjusted buffers at 18 h post-infection, and fused cells imaged using a fluorescent microscope. Using this assay, A/CA09 and B/MASS12 were shown to initiate fusion of 293 T cells at pH 5.6, and cells were completely fused at pH 5.4. A/NC99, A/BOL13, and A/SYD97 initiated cell-to-cell fusion at pH 5.4, and 293 T cells showed complete fusion at pH 5.2. For these latter three viruses, the fusion pH values were approximately 0.1 units higher than those reported using the quantitative virus inactivation assay; however, importantly, the pH stability profile of the three viruses was consistent between assays. These data indicate that the suboptimal VE of A/BOL13 is unlikely to be due to its HA pH stability profile.

3.4. A/H1N1pdm09 A/CA09 shows a greater reduction in virus potency than other 2013–2014 QLAIV strains after heat treatment of Fluenz® nasal sprayers

A further experiment was conducted to confirm a role for HA thermal instability in the reduced VE of A/CA09. To simulate the potential environmental heat exposure during distribution of a number of QLAIV lots in the 2013–2014 season [8], representative commercial nasal sprayers containing QLAIV formulation A/CA09, A/TEX12, B/MASS12, and B/BRIS08, were either exposed to 33 °C for 4 h, or left untreated (control). These sprayers were then held at 4 °C for 12 weeks, typical storage conditions for the vaccine. To assess thermostability slopes and determine the reduction in virus titre in the presence or absence of heat exposure, one sprayer from each treatment group was removed from 4 °C every 7 days, and each strain was titrated by FFA assay. The data obtained from these assays were analysed using a linear regression curve fit statistical model (method detailed in the supplementary information). Fig. 4 shows the virus titre degradation slope values calculated for sprayers from the 33 °C group and the untreated control group. Comparison of treated and untreated degradation slopes for each virus showed that the reduction in A/CA09 potency was significantly more rapid following heat treatment ($p = 0.031$), while A/TEX12, B/MASS12, and B/BRIS08, all showed similar rates of potency reduction in both treatment groups ($p > 0.05$). Therefore, A/CA09 stability was more impacted by the heat treatment than the other strains in this QLAIV formulation.

Despite the more accelerated rate of potency reduction of A/CA09 in the heat-treated sprayer group, this resulted in a virus titre of 6.52 Log_{10} focus forming units (FFU)/dose at the end of the 12-week period. While significantly reduced compared to the control group, this remained within LAIV product specification (7 ± 0.5 Log_{10} FFU/dose) during this time period. However, given the slope, it is possible that this would not have remained true following more prolonged storage. Comparable results were obtained using 50% tissue culture infective dose ($TCID_{50}$) assays (data not shown).

The HA viruses used in these studies is summarised thermal and pH stability data obtained for the panel of LAIV in Table 1 with their known VE. For each virus, the known vaccine efficacy/effectiveness (yes or suboptimal), thermostability at ≥57.5 °C (yes or no), and approximate HA activation pH value is detailed.

4. Discussion

The A/H1N1pdm09 A/CA09 HA protein of LAIV and wild-type viruses has been demonstrated by us, and others, to have poor thermostability [9,10,13]. This has been reported as a likely contributing factor to the reduced VE of this strain in QLAIV, due to a significant correlation observed between reduced LAIV VE against A/H1N1pdm09 viruses and higher outdoor temperatures during LAIV lot unloading at US distributors [8]. Here we carried out a simulated heat exposure of representative nasal sprayers to experimentally confirm this hypothesis. While our data showed that A/CA09 was significantly more susceptible to the heat treatment, the viral potency remained within LAIV product specification (7.0 ± 0.5 Log_{10} FFU/dose). It is worth noting that although the A/CA09 titre was within specification at the end of 12 weeks, it is possible that this would not have remained the same after a longer time period.

However, heat-treated A/TEX12 and B/BRIS08 viruses also demonstrated a degree of degradation over time and their ther-

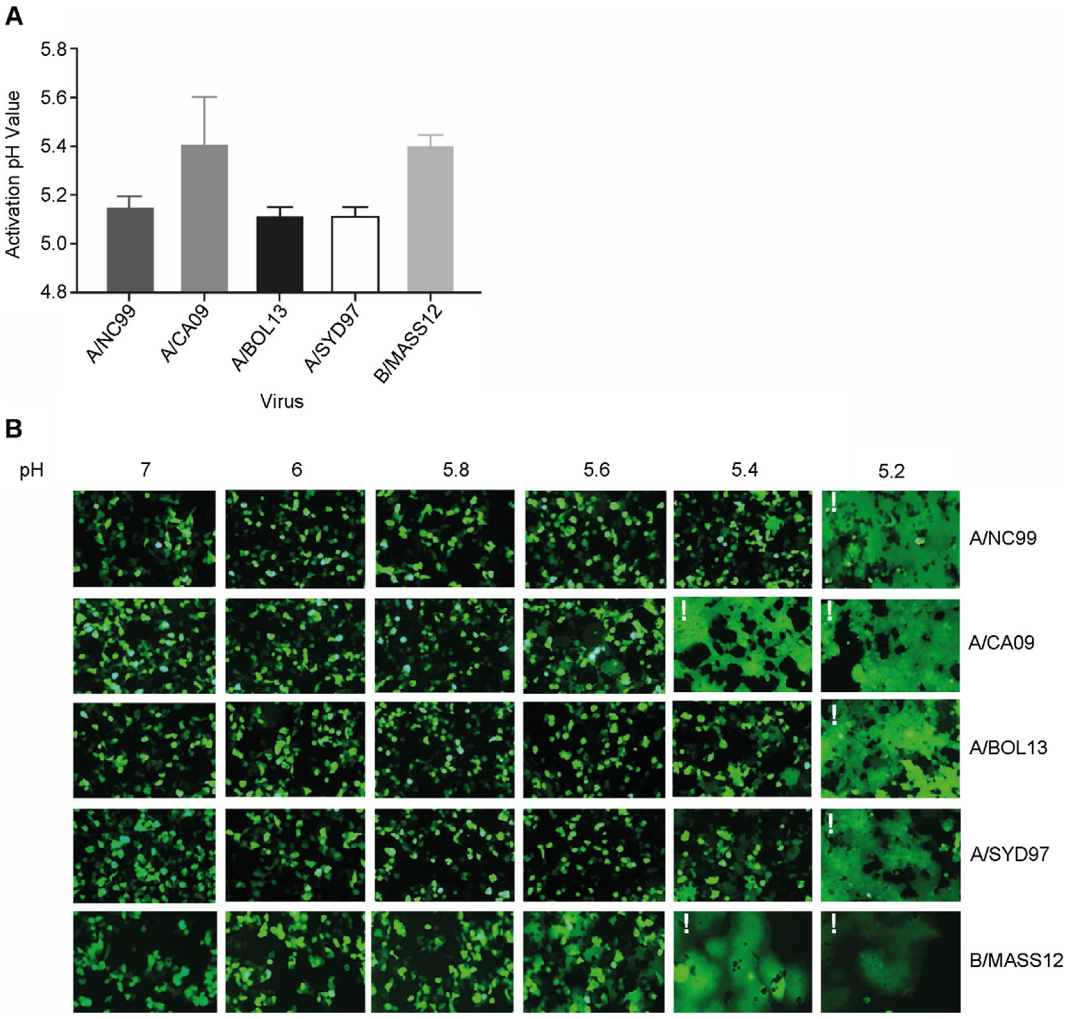

Fig. 3. pH stability of A/H1N1pdm09, A/H3N2, and B-strain live attenuated influenza vaccine viruses. (A) Viruses were incubated with pH buffers ranging from pH 5 to pH 7 and used to infect A549 cells. Cells were fixed and stained for viral NP and with Hoechst stain at 18 h post-infection, and infected cells were quantified using a high content imaging system. Infectivity at each pH was calculated as percentage of the pH 7 control, and the pH value at which 99% of virus infectivity was lost (pH of haemagglutinin [HA] activation) was derived. (B) 293 T cells transiently expressing green fluorescent protein (GFP) were infected with each virus and at 18 h post-infection, were treated with TrypLE to cleave surface expressed HA0. Subsequently, pH buffers ranging from pH 5 to pH 7 were used to activate the viral HA and induce cell-cell membrane fusion. Successful fusion was visualised using GFP fluorescence. White '!' indicates the presence of fused cells. Images are representative of this assay performed in independent triplicates. Images from pH 5 not shown as cells were lysed in those sample wells.

mostability curve slopes were not markedly different from those observed for A/CA09. Additionally, potency reduction of B/MASS12 in the no heat-treatment control group was calculated as + 0.03 Log_{10} FFU/dose over 12 weeks. As it is unlikely that B/MASS12 became more stable after exposure to 33 °C, this may be indicative of the variability of the stability curves obtained. Taken together, these data suggest that while the increased thermal instability of A/CA09 may have played a role in the suboptimal VE of this strain, other factors were likely to have been involved.

Our studies also provided a novel assessment of the expected stability profiles associated with a clinically efficacious LAIV strain. We demonstrate that the clinically effective LAIV strains A/NC99, A/SYD97, and B/MASS12 are more thermostable than A/CA09. We also show that the HA of A/CA09 is more pH sensitive when compared with efficacious influenza A strains, A/NC99 and A/SYD97. As shown in Fig. 3a and 3b, B/MASS12 was similarly pH sensitive to A/CA09, yet was thermostable in both accelerated (Fig. 1a and b) and longer-term (Fig. 4) thermostability assays.

Even though exposure to both increased pH and temperature can cause the irreversible conformational change necessary to expose the fusion peptide and thus activate the viral HA [22–24], the relationship between the two properties may not necessarily be directly correlative between subtypes, but may correlate within certain subtypes. Our data indicate that the two phenotypes may be correlative within the A/H1N1 subtype, but HA thermal and pH stability may have a different relationship in B-Yamagata lineage viruses. Further studies of this type on a wider panel of influenza B viruses would be necessary to fully describe this relationship. This is also in line with others reporting that the pH/temperature relationship is likely to be influenza subtype and strain-specific [39–41].

It is worth noting that for three of the LAIV viruses included in these studies (A/NC99, A/BOL13, A/SYD97), the activation pH values generated by the qualitative cell-cell fusion assay were approximately 0.1 units higher than those reported using the quantitative virus inactivation assay; however, the pH stability profiles of the three viruses were consistent between assays. This is possibly because the cell-cell fusion assay may only require a proportion of surface-expressed HA molecules to become activated for visible fusion to occur. This could provide a more sensitive assessment of HA activation pH than the complete inactivation of viral infectivity.

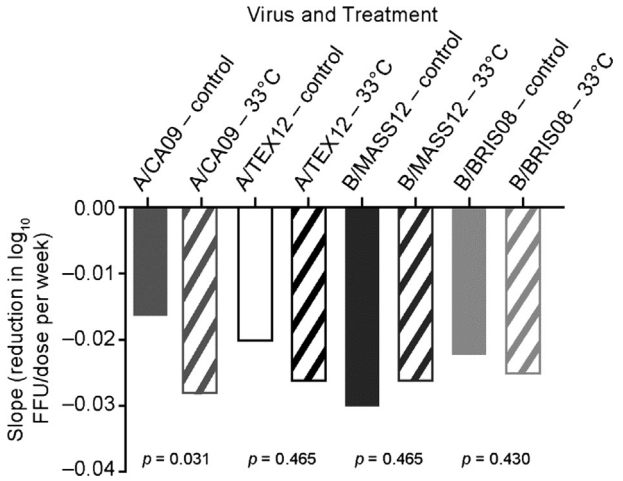

Fig. 4. Long-term thermostability of live attenuated influenza vaccine viruses from 2013 to 2014 season Fluenz® nasal sprayers. The reduction in virus titres of A/CA09, A/TEX12, B/MASS12, and B/BRIS08 viruses in quadrivalent nasal sprayers were measured over 12 weeks after heat treatment at 33 °C for 4 h (striped bars). A control group was also included that received no heat treatment (solid filled bars). Sprayers from both groups were held at 4 °C for 12 weeks and every 7 days the titres of each of the four viruses were measured by focus-forming assay (FFA). A linear regression curve fit model (detailed in the supplementary information) was used to calculate the slopes resulting from change in FFA titre over 12 weeks (y-axis) for each treatment group. P-values comparing the two treatment groups for each virus are shown, with $p < 0.05$ indicating significance.

Here, we demonstrate that A/CA09 is more pH sensitive and heat labile than A/BOL13, the A/H1N1pdm09 virus developed as a replacement for A/CA09 in the 2015–16 season QLAIV. A/BOL13 was isolated 4 years after the first wave of the 2009 H1N1 pandemic, and carries key HA amino acid residues known to have arisen naturally during the evolution of A/H1N1pdm09 viruses in this period, which improved the structural stability of the protein, including P83S, S185T, S203T, I321V, E374K, and S451N [10].

The E374K substitution is one of the most well-documented for its role in maintaining the structural integrity of functional HA trimers by interacting with HA1 residue number 21 via a salt bridge [9,10]; however, other amino acid residues involved in A/H1N1pdm09 HA stability improvement have recently been identified. A stabilising Y7H (HA2) substitution was shown to be required for successful airborne transmission in ferrets [42], and a Y161F (HA1) substitution enhanced IIV virus antigen yield in Madin-Darby Canine Kidney (MDCK) cell cultures, as well as improving thermostability [43]. Assessment of such amino acid residues as determinants of HA stability could prove useful during the development of future A/H1N1pdm09 LAIV strains. It is also worthwhile to note that other influenza virus genes may also play a role in vaccine stability. For example, the matrix gene segment of cold-adapted A/Ann Arbor/6/1960 has been reported to affect temperature and pH sensitivity of pandemic LAIV viruses [44].

It was hypothesised that the lower-than-expected VE of A/BOL13 may have been caused by an over-stabilised HA selected for during the development of a more thermostable LAIV strain. Using thermal and pH stability assays, we have shown that while the HA of A/BOL13 was more stable when compared with A/CA09, it did not appear to be over-stabilised, and demonstrated a stability profile comparable with clinically efficacious A/NC99 and A/SYD97 influenza A viruses. Therefore, we conclude that HA thermal or pH stability was not a root cause for the reduced VE of A/BOL13. The fact that the B-Yamagata lineage virus, B/MASS12, had similar pH sensitivity to A/CA09 and yet was highly clinically effective, while A/CA09 was not, also substantiates this conclusion.

In addition, influenza viruses with high pH sensitivity are frequently considered to be more susceptible to inactivation in the mildly acidic environment of the human upper respiratory tract than those with lower pH sensitivity, rendering them unable to infect cells due to premature, irreversible activation of the viral HA. The fact that a pH–sensitive virus, such as B/MASS12, had good VE indicates that despite this attribute, it was still able to infect and replicate in the cells of the upper respiratory tract. This also argues against pH instability of the HA protein as a hypothesis for the observed reduced VE of A/CA09.

However, given the fact that poor thermostability may have been a contributing factor to the reduced VE of A/CA09, and as pH stability is a property of vital importance to the virus life cycle, stability of the viral H1 HA protein will continue to be assessed during seasonal development of LAIV strains, with the understanding that clinically effective H1N1 strains are thermostable and have an approximate activation pH of 5.1.

Due to the complex nature of live attenuated viral vaccines at both virological and immunological levels, there are a plethora of possible contributing factors to reduced VE, aside from HA stability. Numerous hypotheses have been proposed, including the reduced replicative fitness of A/H1N1pdm09 strains, viral interference and presence of defective interfering particles [16–20]. Reduced viral replicative fitness in the cells of the nasal epithelia offers a plausible root cause [19]. Insufficient replication post-vaccination could render a LAIV strain unable to stimulate a robust immune response. However, efficiency of virus replication is a complex phenotype to dissect, with a multitude of potential effectors affected by changes to the HA and NA genes. These could include: HA/NA balance [45]; altered receptor-binding avidity or specificity of the HA [46]; compatibility of wildtype HA and NA glycoprotein gene segments with the internal A/Ann Arbor/6/1960 gene constellation during genome assembly [47]; or production of excessive non-infectious or defective viral genomes [18]. In addition, in multivalent formulation, a LAIV strain with reduced replication may be subject to competition from other, fitter component viruses [19].

Ongoing work in both human and non-human cell models, as well as in vivo in ferrets, aims to shed light on this hypothesis. The complete findings of these studies will be key to the understanding and continuous improvement of LAIV VE. Based on the ongoing investigative research by AstraZeneca UK, as well as analyses of LAIV effectiveness since the 2009–2010 season, the US ACIP reinstated the recommendation for use of QLAIV for the 2018–2019 season [48]. QLAIV remains an important vaccine option

Table 1
Summary of haemagglutinin (HA) thermal and pH stability, and vaccine effectiveness, of A/H1N1, A/H1N1pdm09, A/H3N2, and B-strain live attenuated influenza vaccine viruses used in this study.

Virus	Subtype	Vaccine effectiveness	Thermostable	HA activation pH
A/NC99	A/H1N1	Yes	Yes	5.1
A/CA09	A/H1N1pdm09	Suboptimal	No	5.4
A/BOL13	A/H1N1pdm09	Suboptimal	Yes	5.1
A/SYD97	A/H3N2	Yes	Yes	5.1
B/MASS12	B-Yamagata	Yes	Yes	5.4

and continues to be used as part of expanding national paediatric influenza vaccination programmes in the UK and Finland.

Acknowledgements

Lauren Parker (LP), Lydia Ritter (LR), Ruben Maeso (RM), and Oliver Dibben (OD) contributed to experimental design. LP, LR and RM all contributed to experimental work, acquisition of data, and subsequent analyses of data. LP and OD were responsible for manuscript writing. Wen Wu (WW) performed all statistical analyses on data obtained from the simulated heat-exposure of Fluenz® nasal sprayer experiments. All authors revised the manuscript critically for important intellectual content and approved the final version to be published. Editorial support including data and fact checking, formatting, proofreading, and submission was provided by Sinead Stewart of Core, London, supported by AstraZeneca according to Good Publication Practice guidelines (Link).

Funding

This study was supported by AstraZeneca UK Ltd.

Declaration of Competing Interest

LP, LR, and OD are employees of AstraZeneca UK Ltd. Helen Bright (HB) and OD are employees and shareholders of AstraZeneca UK Ltd. At the time of data generation, WW was an employee of MedImmune UK Ltd (which at the time was a wholly owned subsidiary of AstraZeneca plc), and is now an employee of Cambridge Data Scientific. At the time of data generation, RM was an employee of MedImmune UK Ltd, and is now an employee of Seqirus Vaccines Ltd. AstraZeneca UK Ltd are the manufacturers of Fluenz®/FluMist® intranasal influenza live virus vaccine.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.06.016.

References

[1] World Health Organisation. Influenza: fact sheet No211. 2003. Available from: [Accessed August 2, 2018]. <http://www.who.int/mediacentre/factsheets/2003/fs211/en/>.
[2] Public Health England. The Winter 2018/19 Flu Vaccination. 2018. Available from: [Accessed October 25, 2018]. <https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/745949/PHE_flu_vaccination_A5_booklet.pdf>.
[3] Centers for Disease Control and Prevention. Prevention strategies for seasonal influenza in healthcare settings. 2018. Available from: [Accessed October 25, 2018]. <https://www.cdc.gov/flu/professionals/infectioncontrol/healthcaresettings.htm>.
[4] Ambrose CS, Luke C, Coelingh K. Current status of live attenuated influenza vaccine in the United States for seasonal and pandemic influenza. Influenza Other Respir Viruses 2008;2:193–202.
[5] European Medicines Agency. Fluenz influenza vaccine (live attenuated, nasal). 2014. Available from: [Accessed August 2, 2018]. <http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Summary_for_the_public/human/001101/WC500103712.pdf>.
[6] Department of Health and Human Services. Clinical review of supplemental biologics license application for MedImmune's quadrivalent live attenuated influenza vaccine (Q/LAIV). 2012. Available from: [Accessed August 2, 2018]. <https://www.fda.gov/downloads/biologicsbloodvaccines/vaccines/approvedproducts/ucm295380.pdf>.
[7] European Medicines Agency. Assessment report: fluenz tetra: common name: influenza vaccine (live attenuated, nasal). 2013. Available from: [Accessed August 2, 2018]. <http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Public_assessment_report/human/002617/WC500158413.pdf>.
[8] Caspard H, Coelingh KL, Mallory RM, Ambrose CS. Association of vaccine handling conditions with effectiveness of live attenuated influenza vaccine against H1N1pdm09 viruses in the United States. Vaccine 2016;34:5066–72.
[9] Cotter CR, Jin H, Chen Z. A single amino acid in the stalk region of the H1N1pdm influenza virus HA protein affects viral fusion, stability and infectivity. PLoS Pathog 2014;10:e1003831.
[10] Yang H, Chang JC, Guo Z, Carney PJ, Shore DA, Donis RO, et al. Structural stability of influenza A(H1N1)pdm09 virus hemagglutinins. J Virol 2014;88:4828–38.
[11] Maurer-Stroh S, Lee RT, Eisenhaber F, Cui L, Phuah SP, Lin RT. A new common mutation in the hemagglutinin of the 2009 (H1N1) influenza A virus. PLoS Curr 2010;2:RRN1162.
[12] Robertson JS, Nicolson C, Harvey R, Johnson R, Major D, Guilfoyle K, et al. The development of vaccine viruses against pandemic A(H1N1) influenza. Vaccine 2011;29:1836–43.
[13] Farnsworth A, Cyr TD, Li C, Wang J, Li X. Antigenic stability of H1N1 pandemic vaccines correlates with vaccine strain. Vaccine 2011;29:1529–33.
[14] Feshchenko E, Rhodes DG, Felberbaum R, McPherson C, Rininger JA, Post P, et al. Pandemic influenza vaccine: characterization of A/California/07/2009 (H1N1) recombinant hemagglutinin protein and insights into H1N1 antigen stability. BMC Biotechnol 2012;12:77.
[15] Caspard H, Mallory RM, Yu J, Ambrose CS. Live-attenuated influenza vaccine effectiveness in children from 2009 to 2015–2016: a systematic review and meta-analysis. Open Forum Infect Dis 2017;4:ofx111.
[16] Ambrose CS, Bright H, Mallory R. Letter to the editor: potential causes of the decreased effectiveness of the influenza A(H1N1)pdm09 strain in live attenuated influenza vaccines. Euro Surveill 2016;21.
[17] Penttinen PM, Friede MH. Decreased effectiveness of the influenza A(H1N1) pdm09 strain in live attenuated influenza vaccines: an observational bias or a technical challenge? Euro Surveill 2016;21.
[18] Gould PS, Easton AJ, Dimmock NJ. Live attenuated influenza vaccine contains substantial and unexpected amounts of defective viral genomic. RNA Viruses 2017:9.
[19] Singanayagam A, Zambon M, Lalvani A, Barclay W. Urgent challenges in implementing live attenuated influenza vaccine. Lancet Infect Dis 2018;18:e25–32.
[20] McLean HQ, Caspard H, Griffin MR, Gaglani M, Peters TR, Poehling KA, et al. Association of prior vaccination with influenza vaccine effectiveness in children receiving live attenuated or inactivated vaccine. JAMA Netw Open 2018;1:e183742.
[21] Russell CJ. Acid-induced membrane fusion by the hemagglutinin protein and its role in influenza virus biology. Curr Top Microbiol Immunol 2014;385:93–116.
[22] Haywood AM, Boyer BP. Time and temperature dependence of influenza virus membrane fusion at neutral pH. J Gen Virol 1986;67(Pt 12):2813–7.
[23] Ruigrok RW, Martin SR, Wharton SA, Skehel JJ, Bayley PM, Wiley DC. Conformational changes in the hemagglutinin of influenza virus which accompany heat-induced fusion of virus with liposomes. Virology 1986;155:484–97.
[24] Kingsley CN, Antanasijevic A, Palka-Hamblin H, Durst M, Ramirez B, Lavie A, et al. Probing the metastable state of influenza hemagglutinin. J Biol Chem 2017;292:21590–7.
[25] Tam JS, Capeding MR, Lum LC, Chotpitayasunondh T, Jiang Z, Huang LM, et al. Efficacy and safety of a live attenuated, cold-adapted influenza vaccine, trivalent against culture-confirmed influenza in young children in Asia. Pediatr Infect Dis J 2007;26:619–28.
[26] Vesikari T, Fleming DM, Aristegui JF, Vertruyen A, Ashkenazi S, Rappaport R, et al. Safety, efficacy, and effectiveness of cold-adapted influenza vaccine-trivalent against community-acquired, culture-confirmed influenza in young children attending day care. Pediatrics 2006;118:2298–312.
[27] Ashkenazi S, Vertruyen A, Aristegui J, Esposito S, McKeith DD, Klemola T, et al. Superior relative efficacy of live attenuated influenza vaccine compared with inactivated influenza vaccine in young children with recurrent respiratory tract infections. Pediatr Infect Dis J 2006;25:870–9.
[28] Fleming DM, Crovari P, Wahn U, Klemola T, Schlesinger Y, Langussis A, et al. Comparison of the efficacy and safety of live attenuated cold-adapted influenza vaccine, trivalent, with trivalent inactivated influenza virus vaccine in children and adolescents with asthma. Pediatr Infect Dis J 2006;25:860–9.
[29] Belshe RB, Edwards KM, Vesikari T, Black SV, Walker RE, Hultquist M, et al. Live attenuated versus inactivated influenza vaccine in infants and young children. N Engl J Med 2007;356:685–96.
[30] McLean HQ, Caspard H, Griffin MR, Poehling KA, Gaglani M, Belongia EA, et al. Effectiveness of live attenuated influenza vaccine and inactivated influenza vaccine in children during the 2014–2015 season. Vaccine 2017;35:2685–93.
[31] Zimmerman RK, Nowalk MP, Chung J, Jackson ML, Jackson LA, Petrie JG, et al. 2014–2015 influenza vaccine effectiveness in the united states by vaccine type. Clin Infect Dis 2016;63:1564–73.
[32] Pebody R, Warburton F, Andrews N, Ellis J, von Wissmann B, Robertson C, et al. Effectiveness of seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2014/15 end of season results. Euro Surveill 2015;20.
[33] Hoffmann E, Neumann G, Kawaoka Y, Hobom G, Webster RG. A DNA transfection system for generation of influenza A virus from eight plasmids. Proc Natl Acad Sci U S A 2000;97:6108–13.
[34] Chen Z, Wang W, Zhou H, Suguitan Jr AL, Shambaugh C, Kim L, et al. Generation of live attenuated novel influenza virus A/California/7/09 (H1N1) vaccines with high yield in embryonated chicken eggs. J Virol 2010;84:44–51.
[35] Jin H, Lu B, Zhou H, Ma C, Zhao J, Yang CF, et al. Multiple amino acid residues confer temperature sensitivity to human influenza virus vaccine strains

(FluMist) derived from cold-adapted A/Ann Arbor/6/60. Virology 2003;306:18–24.
[36] Hoffmann E, Mahmood K, Chen Z, Yang CF, Spaete J, Greenberg HB, et al. Multiple gene segments control the temperature sensitivity and attenuation phenotypes of ca B/Ann Arbor/1/66. J Virol 2005;79:11014–21.
[37] Cheng X, Zengel JR, Xu Q, Jin H. Surface glycoproteins of influenza A H3N2 virus modulate virus replication in the respiratory tract of ferrets. Virology 2012;432:91–8.
[38] Huber PJ, Ronchetti EM. Robust statistics. second ed. Hoboken, New Jersey: John Wiley & Sons, Inc; 2009.
[39] Galloway SE, Reed ML, Russell CJ, Steinhauer DA. Influenza HA subtypes demonstrate divergent phenotypes for cleavage activation and pH of fusion: implications for host range and adaptation. PLoS Pathog 2013;9:e1003151.
[40] Costello DA, Whittaker GR, Daniel S. Variations in pH sensitivity, acid stability, and fusogenicity of three influenza virus H3 subtypes. J Virol 2015;89:350–60.
[41] Scholtissek C. Stability of infectious influenza A viruses at low pH and at elevated temperature. Vaccine 1985;3:215–8.
[42] Russier M, Yang G, Rehg JE, Wong SS, Mostafa HH, Fabrizio TP, et al. Molecular requirements for a pandemic influenza virus: An acid-stable hemagglutinin protein. Proc Natl Acad Sci U S A 2016;113:1636–41.
[43] Wen F, Li L, Zhao N, Chiang MJ, Xie H, Cooley J, et al. A Y161F hemagglutinin substitution increases thermostability and improves yields of 2009 H1N1 influenza a virus in cells. J Virol 2018;92:e01621–e1717.
[44] O'Donnell CD, Vogel L, Matsuoka Y, Jin H, Subbarao K. The matrix gene segment destabilizes the acid and thermal stability of the hemagglutinin of pandemic live attenuated influenza virus vaccines. J Virol 2014;88:12374–84.
[45] Gaymard A, Le Briand N, Frobert E, Lina B, Escuret V. Functional balance between neuraminidase and haemagglutinin in influenza viruses. Clin Microbiol Infect 2016;22:975–83.
[46] Edinger TO, Pohl MO, Stertz S. Entry of influenza A virus: host factors and antiviral targets. J Gen Virol 2014;95:263–77.
[47] Giese S, Bolte H, Schwemmle M. The Feat of Packaging Eight Unique Genome Segments. Viruses 2016:8.
[48] Grohskopf L, Sokolow LZ, Fry AM, Walter EB, Jernigan DB. Update: ACIP recommendations for the use of quadrivalent live attenuated influenza vaccine (LAIV4) — United States, 2018–19 Influenza Season. MMWR Morb Mortal Wkly Rep 2018;67:643–5.