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Letter to the Editor

Ladder Distribution of LAIV Reassortment between Vero Adaption Attenuated Strain and Wide Type Highly Virulent Influenza Virus Strain

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Abstract

It described the "ladder distribution" of live attenuated influenza vaccine (LAIV) reassortment between Vero cells adaption attenuated strain and wide type-high virulent influenza virus strains by the reverse genetics approach. That gene constellation could save the immunogenicity (imm) of the parental strain and make new reassort virus strain attenuation (*att*) characteristics for further vaccine using, rapidly, not only seasonal vaccine but also pandemic flu vaccine.

Keywords: ***reassortment virus; influenza virus; suitable gene constellation; attenuated.***

Dear Editor,

Herein, we report the "ladder distribution" of live attenuated influenza vaccine (LAIV) reassortment between Vero cells adaption attenuated strain and wide type-high virulent influenza virus strains by the reverse genetics approach. In the era of application of reverse genetics to choose influenza virus genomes, the suitable gene constellation identified in the field of high yield reassortant virus in (*hyv*) Vero cells, attenuation (*att*) characteristics and immunogenicity (imm) of the parental strain by different gene segments in vivo.

This finding might be a of great practical benefit to modify reassortment method with Vero cell adaption (*va*) parental strain and wide type (*wt*) virus for improved growth of vaccine 'seed' viruses, which could help influenza disease prevention and intervention initiatives.

We employed the reverse genetics technology to combine eight gene fragments from the A/Yunnan/1/2005(H3*va*) [1] used as *hyv* and *att* strain donor strain and A/Jilin-Chaoyang/16/2016 (H3*wt*) used as wide type highly virulent influenza virus strains, which finally prepared 36 reassortment virus strains by specific gene fragment order. In this study, using mice weight changes and viral loading on the lower respiratory tract to decide the attenuation characteristics; using virus titter changes serially passaged eighteen times on Vero cells to decide the Vero cells high yield characteristics; using the neuraminidase inhibition (NI) and hemagglutination inhibition (HI) titter in the nasal immunized mice serum to decide the immunogenicity characteristics.

Based on our research, through experiments, it had been confirmed that the use of the "4+4, 5+3 or 6+2" gene constellation have meaningful for reassortment with *hyv* and *wt* influenza virus strains in the future, which was displayed on the "ladder distribution" (**Figure 1**).

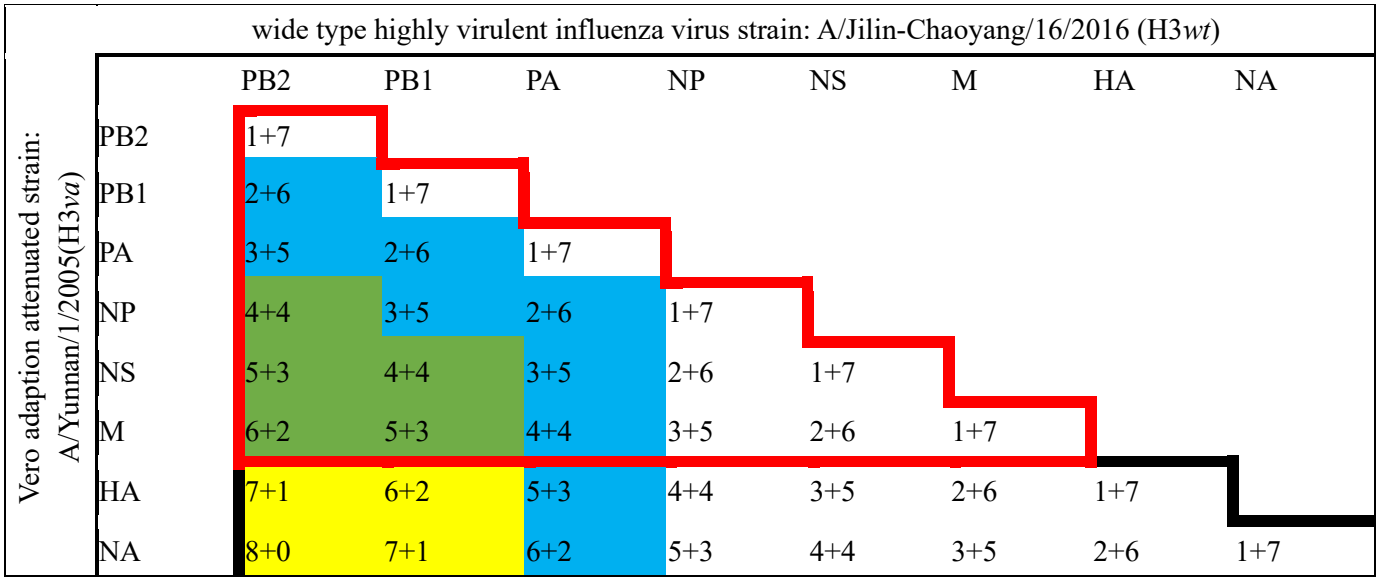

Figure 1: The Ladder distribution of LAIV reassortment between Vero adaption and virulent influenza strain.

Currently there are two techniques to generate HYRs, the classical reassortment method and the reverse genetics approach, which had been illustrated by WHO with the technical road-map [2]. In the classical method, HYRs are generated by co-inoculation of two viruses in ovo (*hyv* donor and *wt* virus), then screening the growth property virus strain after antibody (anti-donor) inhibition. Theoretically, there were 256 reassortant new virus strains need to evaluate, which still exist different probability of some combinations. So, these screening task was a huge project for some laboratory department. Furthermore, the potential bio-safety issues need to be considered when using virulent strains [3,4].

On the other side, the reverse genetics approach provides a suitable practice to reassort the targeted virus strain by using the plasmid system [5,6]. Now, the defined gene constellation (donor+*wt*) become a core issue when using the plasmid system [7,8].

Based on this technology, we constructed and identified the influenza plasmid pool, imparting high yields to candidate vaccine viruses in Vero cell at low temperature [9]. This extensive influenza practical plasmid pool would produce cell culture-derived influenza vaccines (CCIV), within a short time period. We had found that the 6+2 ratio was suitable for some high homology of HA gene, but 5+3 ratio was suitable for some particular strains, by genetic alignment. But in the selection of gene segments, there are still many details that need more research.

Honestly, the A/Puerto Rico/8/1934 (PR8) currently has been widely studied as donor strain in the worldwide, with characteristics of high yield in chicken embryo. Ph.D. Chen considered it have a fixed gene constellation, generally 6+2 (six internal genes derived from PR8 and the genes for the two surface glycoproteins, HA (either native or modified) and NA from the *wt* virus) [6]. Even, Ph.D. Adam Johnson identified the three different PR8 virus lineages as donor viruses had imparted high hemagglutinin yields to candidate vaccine viruses in eggs [10].

Additional, HA gene sequence was considered as a key of its virulent or attenuated characteristics [11], and the PA gene mutation could decrease pathogenicity in chicken embryos and increase the yield of reassortant candidate vaccine viruses [12].

And the advantage of cell culture-derived influenza vaccines (CCIV) had been discussed more and more previously [9,13]. The CCIV permit more rapid vaccine production, are easier to scale up, result in less-adapted mutations, and cause no allergic response-are promising alternative choices for influenza vaccine production [14]. Now, the quadrivalent inactivated influenza virus vaccine produced using the Madin Darby canine kidney cell line has been approved in the EU (Flucelvax® Tetra) and USA (Flucelvax Quadrivalent®; QIVc hereafter) for the prevention of influenza in adults and children [15].

On the other side, the advantage of Vero cells derived vaccine had been discussed by our previous study [1,9]. However, PR8 could not grow efficiently in Vero cells. And there was still some distance between Vero and MDCK cells derived CCIV.

Regarding of Vero cells adaption attenuated virus [9], there was still limitation research on this field, especially, which one fixed gene constellation when using reverse genetics approach to reassort with highly virulent *wt* virus.

Firstly, eight gene fragments of the experimental influenza virus were arranged in a specific order. By the details, the donor strains were arranged on the ordinate, and the parental strain were arranged on the abscissa. The ribonucleoprotein (RNP) related genes were in front, and antigen-related genes (HA and NA) were behind.

Then reassortant viruses were generated from plasmids by a reverse genetics approach as described previous publication [10]. We prepared 36 new reassortant strains virus. Because candidate vaccine viruses (CVVs) must contain genes for the parental surface glycoproteins, HA and NA, we circled its imm gene constellation with a red box in the ladder, with 21 reassortant strains.

Secondly, it need analyzed their attenuation characteristics (*att*) by mice weight changes and viral loading on the lower respiratory tract. And the results were circled the *att* gene constellation with bluen shading. After immunization of mice with virus concentration of 10^6 $TCID_{50}$ in 50μL volume [16], the survival rate of wild type strain mice was 60%, but that of donor strain and new reassortant strains was 100%, during the two-week observation period.

Their body weight was weighed and recorded for 14 consecutive days. The results showed that the maximum weight loss of the immunized mice in each reassortant strain group did not exceed wild strain group, regardless of one or more RNP gene fragment of parental attenuated strain, their weight loss rate was under 65% of the rate of *wt* group.

After three days of immunization, 6 mice of each group were sacrificed, and their nasal and lungs were taken anatomical separation for the tissue viral loading assay. It could find that obvious three main trends, as attenuation (stains circled in blue shading), moderate virulent (stains circled in blue shading in red box) and high virulent strains (the parental virus).

The wild strain had high virulent characteristics, which could proliferate in both upper and lower respiratory tract, and make the viral loading was $10^{7.9}$ $TCID_{50}$ and $10^{7.7}$ $TCID_{50}$, respectively, in nasal and lung tissues. However, the viral loading were $\leq 10^4$ $TCID_{50}$ in nasal and $\leq 10^2$ $TCID_{50}$ in lung for these *att* strains, it was $\leq 10^6$ $TCID_{50}$ in nasal and $\leq 10^4$ $TCID_{50}$ in lung for moderate virulent stains.

The next core issue was the Vero cell adaption, or high yield reassortant in Vero cells. We cultured these new reassortant virus, the donor virus and the parental virus in Vero cells at 33℃, respectively, from the 1st to 18th passages in parallel testing. Then, we calculated their MOI in Vero cells. The reassortant virus strains with specific gene constellation circled in yellow or green shading, could be stably serial passaged in Vero cells with high-yield production, similar to donor strain, especially from the tenth generation. However, the wild-type virus could not be serially passaged in Vero cells which declined from the 2nd generation. And other gene constellation by other color shading could not maintain the virus titter with MOI above 10^6 per ml for five consecutive passages.

Finally, when circled all above testing results on the ladder, it could draw the five useful gene constellation circled in green shading. The combination of genes from attenuated strains and virulent strains should be accurate as illustrated in the **Figure 1**. These five gene constellation could generate new reassortant virus with *att*, *hyv* and imm characteristics. In the meantime, the immune effect of them were also determined by HI and NI testing, which was consistent with our previous research [1,9,17,18].

In summary, this study identified important differences between different gene constellation. And this ladder distribution broadens the applicability of influenza reverse genetics, which could be used both for production of seasonal vaccines and for a rapid global vaccine response during a pandemic, if the reassortment efficiency of the traditional 6+2 constellation had limited.

Abbreviations

live attenuated influenza vaccine: LAIV
Tissue culture infective half dose: TCID50
hemagglutination inhibition: HI
neuraminidase inhibition: NI
ribonucleoprotein: RNP
A/Puerto Rico/8/1934: PR8
candidate vaccine viruses: CVVs
cell culture-derived influenza vaccines: CCIV
high yield reassortant in Vero cells: hyv
Vero cell adaption: va
wide type: wt
attenuated strain: att
high virulent strains: hv
immunogenicity of the parental strain: imm

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Competing interests

There were no competing interests in this study.

Consent for publication

All authors provide consent for this publication.

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