*Bulgarian Chemical Communications, Volume 49, Number 1, (pp. 161 – 170) 2017*

# Optimization of cell culture for H9N2 subtype AIV and establishment of high-yield cell strain

Yu-Jian Lu[1,2,3], Xin-Ming Wu[4], Yu Guan[4], Song-Lin Zhang[4], Jin-Liang Wang[4], Jiu-Xia Zhao[5], Wen-Yu Han[2], Zhi-Qiang Shen[1,4,5*]

[1]*Postdoctoral Program,* [4]*Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou, China*
[2]*Postdoctoral Program, Jilin University, Changchun, China.*
[3]*Department of Life Sciences, Binzhou University, Binzhou, China.*
[5]*Shandong Lvdu Biological Technology Co., Binzhou, China.*

Received October 10, 2016; Revised February 10, 2017

It has become the direction and trend of influenza vaccine production by cell culture. This study aimed to establish the key technologies of the cell culture of H9N2 subtype AIV (avian influenza virus). Results showed that during the culture of the H9-4 isolate in MDCK (Madin-Darby canine kidney) cell lines, when the inoculation amount was $10^{-3}$~$10^{-4}$ with 0.5 μg·mL$^{-1}$ TPCK (tosyl phenylalanyl chloromethyl ketone)-trypsin, 40 mmol·mL$^{-1}$ HEPES (hydroxyethyl piperazine ethanesulfonic acid) or 3 mmol·mL$^{-1}$ Gln (glutamine) in the maintenance media, the proliferation of AIV was excellent at 96~108 hpi (hour post inoculation). Moreover, the supplement of 0.3 μg·mL$^{-1}$ TPCK-trypsin could increase the titer of the virus to a certain extent. The chicken *St3gal*Ⅰ gene was cloned and transfected into MDCK cell lines. By screening, 1 strain of highly-expressed *St3gal*Ⅰ monoclonal cell strain S8 was obtained, of which the α-2,3 linked receptor abundance was evidently increased. On S8 cells, the proliferation of H9-4 was significantly increased. The results confirmed that the infection sensitivity and replication capacity of the isolates of H9N2 subtype AIV were further improved by increasing the receptor abundance on the host cell surface.

**Key words:** H9N2 AIV, MDCK, TPCK-trypsin, *St3gal*Ⅰ gene, α-2,3 linked receptor.

## INTRODUCTION

H9N2 subtype avian influenza is a low pathogenic infectious disease of poultry caused by the AIV [1,2]. In 1966, H9N2 subtype AIV was isolated from the turkeys in the United States for the first time [3]. Since 1997, the subtype AIV has been in the epidemic in birds or poultry around the world [4-6]. H9N2 subtype AIV is a type of low pathogenic AIV (LPAIV), but it can cause the decrease of egg production rate, immune suppression, as well as the mixed infections with other viruses or bacteria, hence huge economic losses were led to the poultry industry [7,8]. In recent years, there have been world-wide incidents of human infections of H9N2 subtype AIV many times, indicating that the virus could increase its gene mutations and recombinant opportunities with other subtypes of influenza viruses because of antigenic drift and antigenic shift, which would result in continuous discoveries of new types of AIV [9-11]. Therefore, H9N2 subtype AIV is a big potential threat to human health.

According to the prevention practices of AIV in China and internationally, the vaccine is the main measure to prevent the avian influenza outbreak and to avoid huge losses. Current production of influenza vaccines for human and animals is mainly the method of embryonated eggs [8]. Despite some effective purification processing, this method still has many disadvantages. For example, the influenza viruses isolated in embryonated eggs often happen to antigenic variations after continuous passages, this method requires high labor intensity, huge amounts of embryonated eggs, sometimes tedious purification processes, and there are also contamination problems caused by exogenous virus [12-15]. The influenza pandemic and the continuous new strains of such influenza virus call for an urgent need to establish a new method of vaccine production. With the improvement and wide application of animal cell culture techniques, the vaccine production using animal passage cells has become an inevitable trend. Compared with the method of embryonated eggs, the cell-culture-based production of influenza virus is economical, convenient and reproducible with stable quality, the mass production is easy to carry out, and the antigenicity is closer to natural strains, therefore the immune effects are quite reliable [15-19]. Currently, the establishment of the flexible and effective cell-culture-based influenza vaccine production platform has become the target of numerous producers. In the vaccine production process, the cell-culture-based production of influenza virus also has disadvantages including poor replication and low yield. In order to increase the yield, it is necessary not only to optimize the conditions for the proliferation of influenza virus, but also to thoroughly study the mechanism of the replication of influenza virus in the host cells. The

To whom all correspondence should be sent:
E-mail: bzshenzq@163.com

hemagglutinin (HA) constitutes the most important surface antigens of H9N2 subtype AIV [20,21]. Cell surface receptors and HA protein receptor binding sites are the two most important factors of the influenza viruses host specificity. There are two common influenza virus receptors: the sialic acid α-2,3 of D-Galactose (SA-alpha-2,3Gal), and the sialic acid α-2,6 of D-Galactose (SA-alpha-2,6Gal) [22-24]. Most AIV preferentially binds to the receptor of SA-alpha-2,3Gal receptors, while human influenza virus mainly binds to the receptor of SAα-2,6Gal [25-27]. The infection of influenza virus is mediated by the sialic acids on the cell surface. Sialic acids usually bind with the 2nd from the bottom at the end of N terminal of the glycans, mainly Galactoses, in the way of α2, 3 or α2, 6 glycosidic bonds [6,28,29]. Under the effects of Sialyl transferases (STs), sialic acids then bind with the terminals of glycoproteins and glycolipids [30-32]. Therefore, it is a potential approach to increase the cell surface receptor abundance through transgenic technology to improve the effect of virus multiplication.

Given that H9N2 subtype AIV is a kind of LPAIV, it hasn't attracted much attention, hence related researches have lagged behind with very inadequate prevention methods. Although vaccination is an effective means to prevent the epidemics of the LPAIV, currently the production of such vaccine takes long preparation period, with low yields and high costs. The main reasons are that the *in vitro* proliferation of H9N2 subtype AIV is very difficult and that the cell culture technology is not mature enough. The goal of this study is optimizing of cell culture conditions of H9N2 subtype AIV, and screen the cell strains with high yields by genetic engineering technologies, so as to try and establish the key technologies and production platform for the cell culture of H9N2 subtype AIV, as well as to provide technical support for the prevention and control of the avian influenza.

## MATERIALS AND METHODS

### *Virus strains, cell lines, bacterial strains and plasmids*

H9N2 subtype AIV local isolates including H9-1, H9-2, H9-3, H9-4, H9-5, H9-6 and H9-BX, and cell lines including MDCK, Vero (Africa green monkey cell ), DF-1 (a continuous cell line of chicken embryo fibroblasts), BHK (Baby hamster kidney), Vero-E and CEF (Chick embryo fibroblast) cells were all provided by Shandong Lvdu Biological Technology Co. Ltd. pCI-*neo* plasmid and DH5α bacterial strain were preserved by Shandong Binzhou Animal Science and Veterinary Medicine Academy. And pMD18-T plasmid was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd.

### *Main reagents*

$T_4$ DNA ligase and restriction endonuclease were purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. Plasmid extract kit and gel extraction kit were purchased from Tiangen Biotech (Beijing) Co., Ltd. Reverse transcription kit, *Taq* DNA polymerase, DNA extraction kit, RNA extraction kit, G418, DMEM cell culture media, TPCK-trypsin, Lipofectamine 2000 Regeant were purchased from Invitrogen (Thermo Fisher Scientific), Fetal Bovine Serum (FBS) was purchased from Gibco (Thermo Fisher Scientific), the primers were produced by Shanghai Generay Biotech Co., Ltd. 1% chicken red blood cell suspension was prepared by blood collection from SPF adult male chickens.

### *Optimization of the conditions of AIV proliferation*

The H9-1, H9-3, H9-4 and H9-5 isolated virus strains were inoculated into the embryonated eggs, the allantoic fluid was harvested after 72 hours, and used as a source of the experimental virus below. The single-layer cells grown on six-well plates (MDCK, Vero, DF-1, BHK, Vero-E and CEF cells) were washed with PBS twice, and then were inoculated with these viruses (H9-1, H9-3, H9-4, and H9-5) at different dilution levels ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$ , and $10^{-6}$). After different time lengths of absorption (0, 30, 60, 90 and 120 min), TPCK-trypsin at different concentrations (0, 0.125, 0.25, 0.5, 0.75, and 1.0 $\mu g \cdot mL^{-1}$), trypsin at different concentrations (0, 2, 5, 8, 10, and 12 $\mu g \cdot mL^{-1}$), serum at different concentrations (0, 0.2, 0.5, and 1.0%), HEPES at different concentrations (0, 5, 10, 20, 30, 40, and 50 $mmol \cdot L^{-1}$), Gln at different concentrations (0, 1, 2, 3, 4, and 5 $mmol \cdot L^{-1}$) or BSA at different concentrations (0, 0.1, 0.2, 0.3, 0.4, and 0.5%), were added into the maintenance media. The supernatant was harvested at different time points (24, 36, 48, 60, 72, 84, 96, 108, and 120 h), and the routine micro-hemagglutination test was conducted to measure the HA titer of the virus. At 24, 48 or 72 h after the inoculation, 0.3 or 0.5 $\mu g \cdot mL^{-1}$ TPCK-trypsin could be re-added to the maintenance media [33].

### *Virus strain stability assay*

The cells in the form of dense single layers were washed with PBS twice, and then were inoculated by $10^{-3}$ diluted H9-4 embryonated egg virus. After 60 min of absorption, the maintenance media containing 0.5 $\mu g \cdot mL^{-1}$ TPCK-trypsin was added. And at 96 hpi the cell viruses were harvested [34]. During the 10 passages in MDCK cells, the HA titer of each passage was tested [35].

*Gene cloning and vector construction*

Based on the sequencing imformation of chicken sialytransferase gene *St6gal* Ⅰ, the primers *St3gal* Ⅰ LP: 5'-GGGGAATTCATGGTCACCGTCAGGAAA-3', and *St3gal* Ⅰ RP: 5'-GGGGTCGACTCATCTGCCCTTGAAAAAT-3' were designed [31]. The 5' terminals of the primers were added with restriction enzyme cutting sites *EcoR* Ⅰ and *Sal* Ⅰ. With the SPF embryonated egg of 12 d, RNA was extracted to produce reverse transcription cDNA. The high-fidelity *Taq* enzyme was used for the amplification of *St3gal* Ⅰ, then the product was lined with pMD18-T and sequenced. The restriction enzyme cutting of pMD18-T-*St3gal* Ⅰ and pCI-*neo* plasmids were performed with *Sal* Ⅰ-*EcoR* Ⅰ, and the extracted target segements were linked, then the eukaryotic expression vector pCI-*neo*-*St3gal* Ⅰ was then established.

*MDCK cell transinfection and cloned culture*

Lipofectamine 2000 was used to transfer pCI-*neo*-*St3gal* Ⅰ plasmids into MDCK cells, and the transgenic cell lines were acquired after 500 μg·mL$^{-1}$ G418 (neomycin) screening (according to the specifications). The transgenic cells were diluted to the concentration of 50/100 μL. Then 2 μL cell suspension was added to 96-well plate, with the re-addition of 100 μL DMEM culture media containing G418. The single cell formed clonal clusters after 10 d culture. After the consumption of the cells, the culture was continued. With 90% confluence, the cells were transfered into 24-well plate to expand the culture [31,36].

*Quantitative real time PCR*

The total RNA of the stable cell strains were extracted and then used to produce cDNA by reverse transcription kit. With *β-actin* gene of MDCK cells as the internal reference, the sequences of the primers were: *β-actin* LP: 5'-CCTCTATGCCAACACAGT-3', *β-actin* RP: 5'-GTACTCCTGCTT GCTGAT-3'. The expression of *St3gal* Ⅰ in transfected cells were measured by qRT-PCR (Quantitative real time PCR) assay. The primers for the amplification of *St3gal* Ⅰ segment were: *St3gal* Ⅰ LP: 5'-ACGAATCAGATGTTGGGAGC-3' and *St3gal* Ⅰ RP: 5'-CATTCACCTCATCGCATA-3'. The qRT-PCR assay was performed by LightCycler®480 SYBR Green Master Kit by Roche. The results and date were analyzed by LightCycler®480 software and Excel 2003 [37].

*FCM assay*

The control and the stable transfected cell strains were put into the 6-well plate evenly. At the cell density of 90%, the cells were consumed and collected, then washed 2 times by PBS containing 10 mmol·L$^{-1}$ Glycine, and then washed 1 time by Buffer 1 (50 mmol·L$^{-1}$ Tris-HCl, 0.15 mol·L$^{-1}$ NaCl, 1 mmol·L$^{-1}$ $MgCl_2$, 1 mmol·L$^{-1}$ $MnCl_2$ and 1 mmol·L$^{-1}$ $CaCl_2$, pH 7.5). The cells were re-suspended by Blocking Solution on ice for 1 h, and then were processed with Maackia amurensisagglutinin (MAA) and blank control for incubation on ice for 1 h. The cell sedimentation was re-suspended by Rhodamine-labeled anti-digoxin secondary Ab for incubation on ice for 1 h. In the end, after 3 times wash with PBS, the products were tested by flow cytometry (FCM) [36].

*$TCID_{50}$ and $EID_{50}$ assay*

The control and the stable transfected cell strains were put into the 96-well plate evenly. At the cell density of 90%, the cells were inoculated with AIV which was continuous 10 times serially diluted. The CPE (cytopathic effect) were observed continuously for 72 h, then the viruses were harvested for HA test. The $TCID_{50}$ (tissue culture infective dose) was calculated by Reed-Muench method. The virus was diluted by saline and inoculated into the allantoic cavity of 10 d SPF chicken. The survival of embryonated eggs was observed continuously for 72 h, then the viruses were harvested for HA test. The $EID_{50}$ (egg infectious dose) was calculated by Reed-Muench method [38].

*Data analysis*

The date were analyzed by Excel 2003 and SPSS 19.0. The significance analysis of the differences among multiple groups were conducted with Duncan's Multiple Range Test. Same letter was defined as insignificant difference, while different letters indicated significant differences on 0.05 level (lowercase letters). The significance of the difference between two average numbers were tested by the t test, $p$ <0.05 was defined as significant difference, $p$ <0.01 was defined as highly significant difference, and $p$ <0.001 was defined as extremely significant difference.

## RESULTS AND DISCUSSION

*The establishment of the key process conditions of cell culture of H9N2 subtype AIV*

*Cell proliferation features of H9N2 subtype AIV*

The *HA* gene sequence of the isolated virus strains was compared with some international reference strains and the representative strains, which showed that these strains were divided into 3 evolved subtypes: representative strain AF156376 (Subtype I), AF156378 (Subtype II), and AF156377 (Subtype III) respectively.

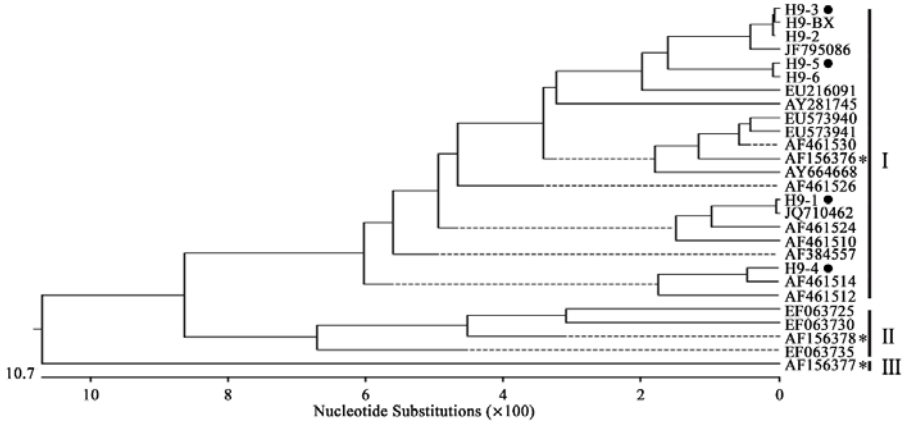


**Fig. 1.** The phylogenetic relationship analysis of 7 isolates compared with *HA* gene.

In this study, 7 strains of H9N2 subtype AIV in the comparisons (including H9-1, H9-2, H9-3, H9-4, H9-5, H9-6, and H9-BX) were all in Subtype I, with the representative strain as A/duck/Hong Kong/Y280/97 (AF156376), indicating that the 7 isolates all belonged to Eurasian strains with closed phylogenetic relationship (Figure 1). According to the locations of the isolates on the phylogenetic tree, H9-1, H9-3, H9-4, and H9-5 were selected as the candidate strains for the production of AIV vaccine.

The 4 selected strains were inoculated to MDCK cells and the hemagglutination values were tested after harvest. The results were shown in Figure 2A. With the same amount of inoculation, the proliferation of H9-4 was the best, with significant differences between the other 3 isolates. This indicated that H9-4 could proliferate easily in the cells with the potential to become a vaccine strain. H9-4 presented different sensitivities to different host cells, and that MDCK cells were the most suitable for the proliferation of H9-4 (Figure 2B). The difference of the sensitivity might be because of the better binding ability of H9-4 to cell surface receptors and the more influenza virus receptors on MDCK cells to facilitate more viruses to absorb and invade. The proliferation of H9-4 was better after 24 h cell culture with the inoculation at $10^{-3}$~$10^{-4}$ (Figure 2C). Too much inoculation dose would accelerated cytopathic changes in the cells, and inhibit the proliferation of the viruses; while inadequate inoculation led to poor proliferation in the cells. During 96~108 hpi, almost all the cells presented CPE, and the titer of the virus was close to the peak value (Figure 2D). With the cell culture time, the titer decreased mainly because the constant nutrient consumption of the media and the deaths of the host cells [39]. The absorption time also influenced the proliferation of AIV: with the absorption time as 60 min, the HA titer of H9-4 was higher (Figure 2E). The HA titer was evidently increased after 2 times of freezing and thawing of cells with $10^{-3}$ dilution of the viruses (Figure 2F). Higher serum concentration could present higher inhibitory effects (Figure 2G). This was because serum not only contained trypsin-resistant substance damaging the proteolysis effects of trypsin, but also had some substances that could cover virus receptors on the cell membrane, thus affecting the virus infection of the cells. Therefore, during the cell culture of H9-4, serum shouldn't be added to the maintenance media.

*The influences of trypsin on the proliferation of AIV*

After the inoculation of MDCK cells, trypsins with different concentrations were added into the maintenance media. The results showed that with the concentrations of TPCK-trypsin and trypsin at 0.5 $\mu g \cdot mL^{-1}$ and 10 $\mu g \cdot mL^{-1}$ respectively, the titer of the virus was at the peak value (Figure 3A and B). Data analysis suggested obvious improving effect of trypsin on the proliferation of AIV, and the effect of TPCK-trypsin was better than the ordinary trypsin. In addition, 24 h or 48 h after the inoculation of MDCK cells, the supplement of 0.3 $\mu g \cdot mL^{-1}$ and 0.5 $\mu g \cdot mL^{-1}$ TPCK-trypsin into the maintenance media could both increase the titer to a certain degree, with the better proliferation result of the addition of 0.3 $\mu g \cdot mL^{-1}$ TPCK-trypsin at 48 hpi (Figure 3C).

The influenza virus HA was split by the host protease into the mature HA1 and HA2, which should be the precondition of the cell infection of AIV. However, such protease was lacking in the tissues and cells, the right amount of exogenous trypsin should be added during the proliferation period of AIV, so as to increase the splitting of HA and to enhance the infectivity of AIV [40,41]. But the excessive level of trypsin would cause the early cell shedding and the reduction of the titer, while the inadequate trypsin couldn't have the effects. After the treatment with TPCK, trypsin could present higher activity and better improving influence on the proliferation of the viruses.

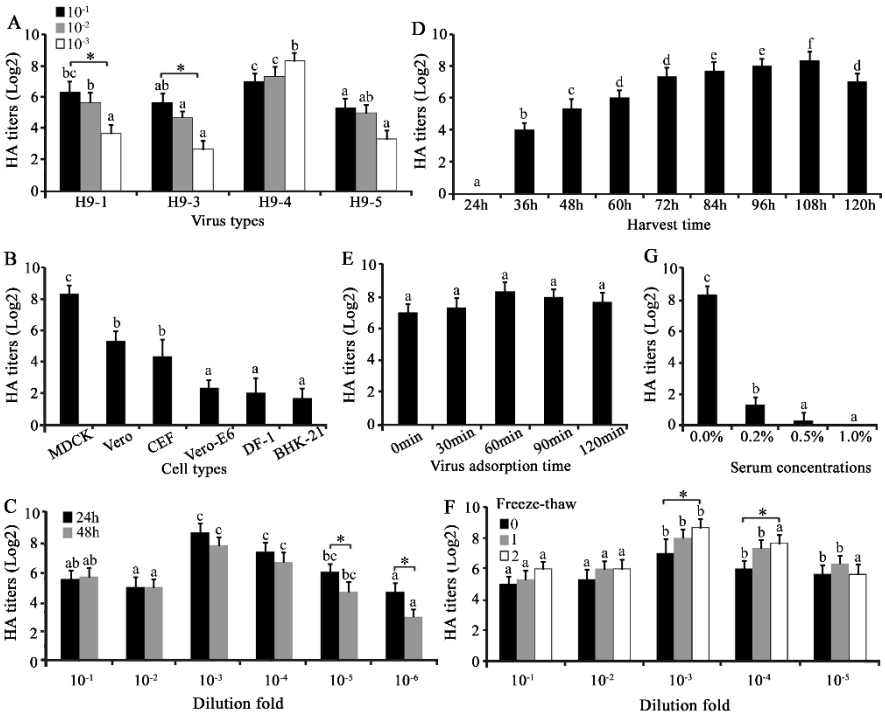


**Fig. 2.** Cell proliferation features of H9N2 subtype AIV. The different isolated virus strains (A), host cells (B), cell culture time and inoculation amount (C), harvest time (D), absorption time (E), freezing and thawing times (F), and serum concentration could influence the proliferation of AIV. The virus used in the other figures was H9-4 isolated strain except fig. 2A. The cell line used in the other figures was MDCK except fig. 2B. Same letter was defined as insignificant difference, while different letters indicated significant differences on 0.05 level. An asterisk (*) represented $p < 0.05$, two asterisks represented $p < 0.01$, and three asterisks represented $p < 0.001$.

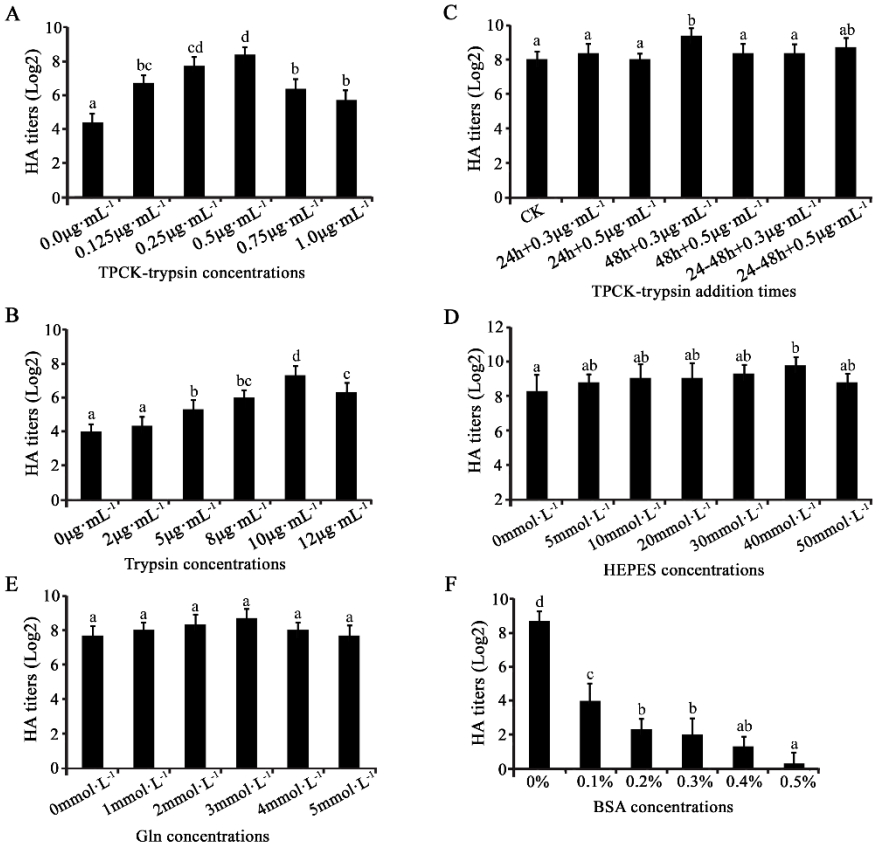


**Fig. 3.** Optimization of the cell culture conditions of H9N2 subtype AIV. The different TPCK-trypsin (A), Trypsin (B), TPCK-trypsin addition times (C), HEPES (D), Gln (E), and BSA (F) could influence the proliferation of AIV. The virus strain and cell line used in fig.3 were H9-4 and MDCK respectively. Same letter was defined as insignificant difference, while different letters indicated significant differences on 0.05 level.

*Other factors influencing the proliferation of AIV*

After the inoculation of MDCK cells, the solutions at different concentrations of HEPES, Gln or bovine serum albumin (BSA) were added into the maintenance media. The results showed that the HA titer was the highest with 40 mmol·L$^{-1}$ HEPES with significant differences, that the HA titer was thehighest with 3 mmol·L$^{-1}$ Gln with non-significant differences, and that BSA inhibited the proliferation of H9-4: with higher BSA concentration, the titer was significantly lower (Figure 3D-F).

HEPES, as a hydrogen ion buffer, might maintain the long-term constant pH range; Gln, as the must-have additive in *in vitro* cell culture, was used as an energy source in the synthesis of protein and nucleic acid metabolism. Therefore, adding the right amount of HEPES and Gln in the maintenance media could help maintain normal cell growt. Just like serum, the addition of BSA also led to the obvious reduction of the titer, probably because it occupied the cell-surface receptors.

*Stability of H9-4 isolates*

After 11 continuous passages of H9-4 isolates from embryonated eggs in MDCK cells, the results showed that the HA titer increased gradually and achieved to the peak value at passages F4, and then began to fall (Figure 4A). This is probably because there was a gradual process of adaptation for the proliferation of AIV in the host cells. But the increased trypsin inhibitor accumulation level elevated with the passages and caused the inhibition to the proliferation, or it was also possible that the abundance of the α-2,3 linked receptors was still low on MDCK cells [39,42].

*Analysis on the key factors influencing the proliferation of AIV*

H9-4 isolate was inoculated into MDCK and Vero cells accordingly, with the addition of 0.5 μg·mL$^{-1}$ TPCK-trypsin, 40 mmol·L$^{-1}$ HEPES or 3 mmol·L$^{-1}$ Gln. It could be seen that without TPCK-trypsin, there was hardly any proliferation of virus in Vero cells whether or not with the addition of HEPES or Gln. For MDCK, it was found that without TPCK-trypsin, there was poor proliferation of AIV; and that with TPCK-trypsin, the HA titer increased significantly, and improvement of the proliferation if with the addition of HEPES or Gln (Figure 4B). The results above indicated that TPCK-trypsin was the most important factor influencing the proliferation of H9-4, and the right amount of HEPES or Gln improved the proliferation of the virus.

In order to better understand the effects of the supplement of TPCK-trypsin, 24 h, 48 h or 72 h after the inoculation of Vero and MDCK cells, 0.3 μg·mL$^{-1}$ TPCK-trypsin was re-added and the HA titer was measured at 96 hpi. The results indicated the re-addition of TPCK-trypsin could improve the proliferation of the virus in both cells, but more evidently in MDCK cells (Figure 4C). The reason might be that with the elevated accumulation of trypsin inhibitor during the replication of AIV, the trypsin in the culture media was inactivated fast, and only the timely addition of TPCK-trypsin could meet the needs of effective proliferation of AIV [17,43,44].

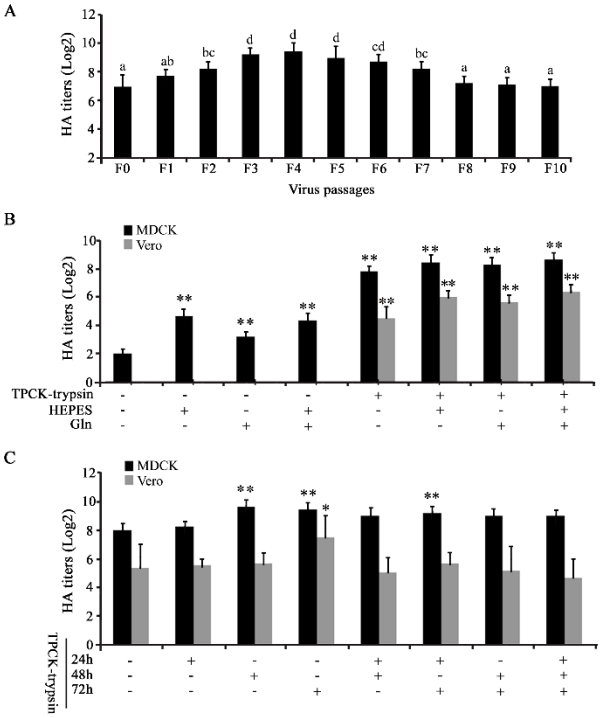


**Fig. 4.** Analysis on stability of H9N2 subtype AIV isolate and the key factors influencing the proliferation of AIV. (A) Stability of H9-4 isolate; (B) Effect of TPCK-trypsin, HEPES and Gln on the proliferation of H9N2 subtype AIV; (C) Effect on the proliferation of H9N2 subtype AIV supplemented with TPCK-trypsin at different time. The virus strain used in fig.4 was H9-4. The cell line used in fig. 4A was MDCK. Same letter was defined as insignificant difference, while different letters indicated significant differences on 0.05 level. An asterisk(*) represented $p$ <0.05, two asterisks represented $p$ <0.01, and three asterisks represented $p$ <0.001.

*Establishment of high-yield cell strain of H9N2 subtype AIV*

*Construction of pCI-neo-St3gal Ⅰ vector and screening of stable transfected cell strains*

In addition to optimizing the conditions of the proliferation of influenza virus, other methods could also be applied, such as reforming the cell lines for the better virus proliferation environment, the higher yield and titer. Influenza virus could

infect the host by the specific binding of HA with the host cell surface sialic acid receptor [25,45]. MDCK cells had both SAα-2,3Gal and SAα-2,6Gal receptors, while the abundance of the SAα-2,3Gal receptors were quite low and caused the low liter of AIV in MDCK cells [46]. The α-2,3-sialyltransferase Ⅰ(ST3Gal Ⅰ) could transfer the sialic acid to the host cell surface in the form of α-2,3 link, and formed the receptor of AIV [31]. Therefore, through the high expression of *St3gal* Ⅰ, the abundance of SAα-2,3 Gal receptors on MDCK cells could be improved.

The SPF chicken *St3gal* Ⅰ gene was cloned and inserted into pCI-*neo* plasmid (Figure 5A). After identification, the expression vector pCI-*neo*-*St3gal* Ⅰwas obtained (Figure 5B). The pCI-*neo* and pCI-*neo*-*St3gal* Ⅰplasmids were used for the transfection of the MDCK cells. Then 30 monoclonal cell strains were selected and picked out. The PCR assay of the genomic DNA indicated only the cell strains with the target genes could present ~1 kb amplification band (Figure 5C). The total RNA of the transfected cells were extracted for RT-PCR assay. The results showed that the stable transfected cell strains could amplified proper bands (Figure 5D). 9 transfected cell strains with good amplification effect were selected for the transcription level detection of *St3gal* Ⅰ with *β-actin* gene as internal reference. The results showed that the *St3gal* Ⅰ expression level in transfected cell strains were higher comparatively (Figure 5E). Combining the results of qRT-PCR, the expression level of *St3gal* Ⅰ gene was the highest in S8 cell strain ($p$ <0.001), followed by S1, S3 and S4 ($p$ <0.01) (Figure 5F). The HA titer analyses showed the better proliferation of H9-4 in S8 cell strain than the control (Figure 5G).

### *Analysis of high-yield cell strain receptor abundance and AIV proliferation effect*

Microscopic observation found that the normal MDCK cells, empty transfected and S1 cells were spindle-shaped, however S8 cells in tight arrangement appeared prismatic-shaped (Figure 6Aa-d). At 72 hpi, the growth of the control cells (uninoculated) were almost normal, with few cell masses and shedding cells (Figure 6Ae). However, the CPE were observed for the cells inoculated H9-4 virus (Figure 6Af~h). By comparison, the cytopathogenic degree of S8 cells was more severe and there were fewer adherent cells. The abundance of α-2,3 linked receptors on the stable transfected cell strains was detected by FCM assay.

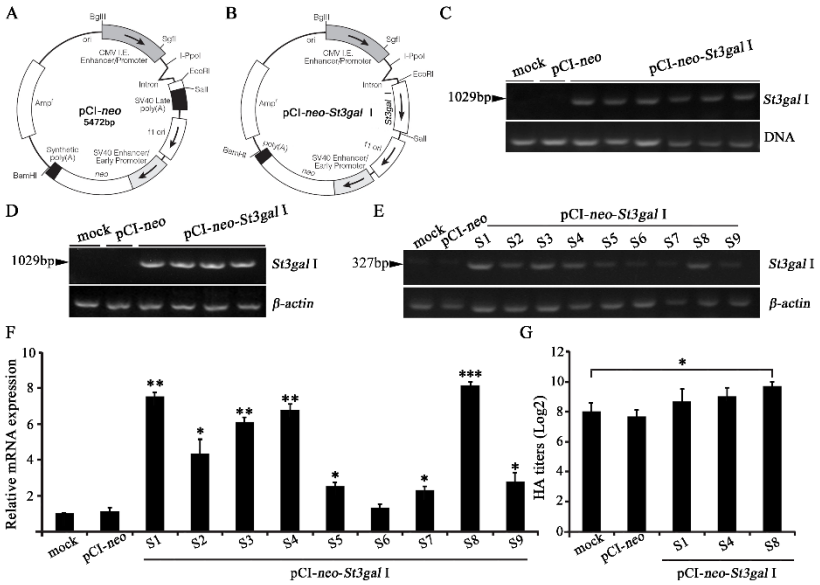


**Fig. 5.** Construction of eukaryotic expression vector and screening of stable transfected cell strains. (A) Schematic illustration of pCI-*neo* expression vector; (B) Schematic illustration of pCI-*neo*-*St3gal* Ⅰ expression vector; (C) PCR assay of stable transfected cell strains; (D) RT-PCR assay of stable transfected cell strains; (E) Detection of the *St3gal* Ⅰ expression level in stable transfected cell strains by RT-PCR; (F) Detection of the *St3gal* Ⅰ expression level in stable transfected cell strains by qRT-PCR; (G) HA titer analyses of stable transfected cell strains by inoculation of H9N2 subtype AIV. In Fig. 5G, the culture conditions of the H9-4 isolate in different MDCK cell lines: the inoculation amount was $10^{-3}$ with the addition of 0.5 μg·mL$^{-1}$ TPCK-trypsin, the harvest time of AIV was at 96 hpi. An asterisk(*) represented $p$ <0.05, two asterisks represented $p$ <0.01, and three asterisks represented $p$ <0.001.

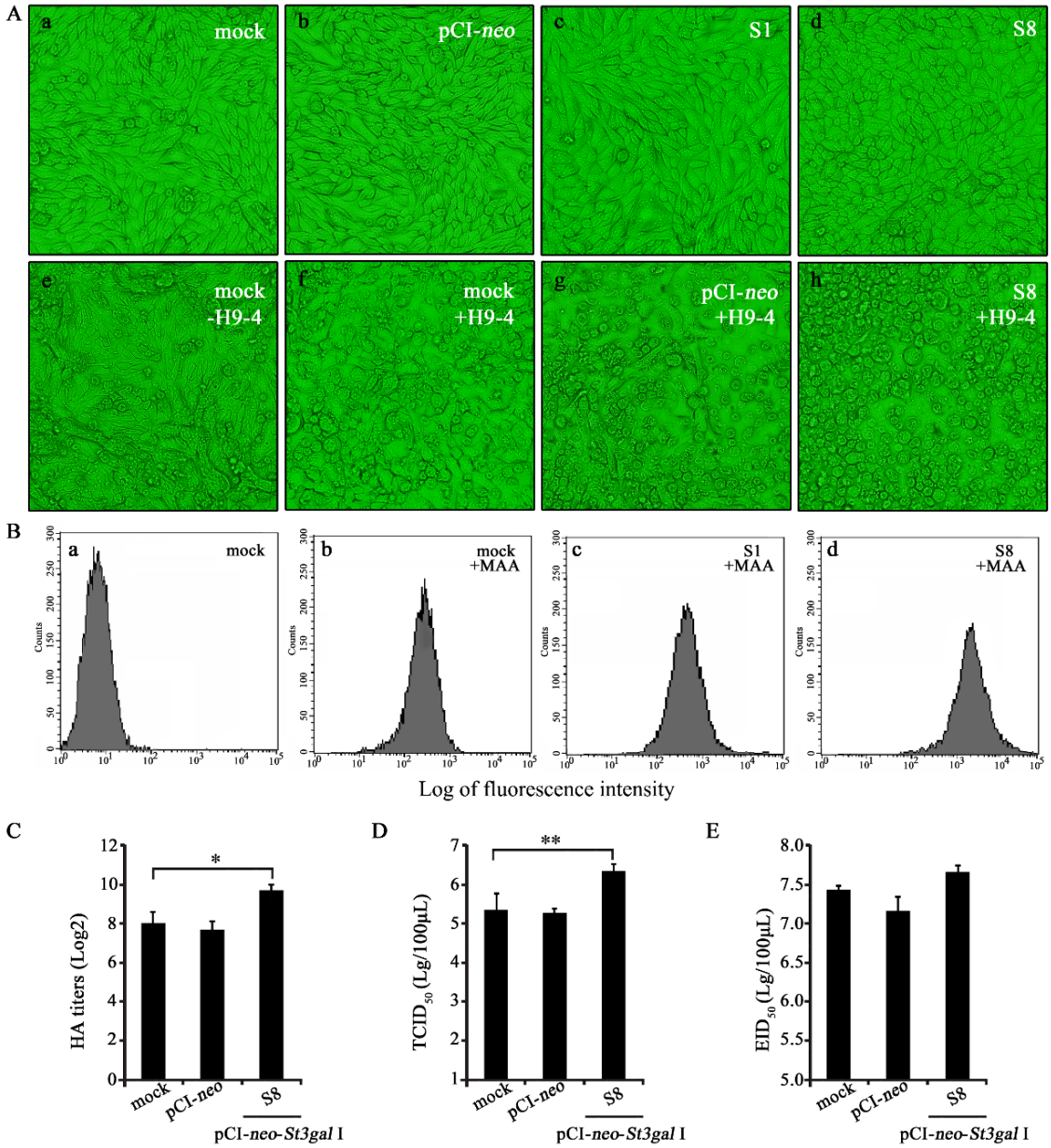


**Fig. 6.** Detection of receptor abundance and AIV proliferation effect. (A) The morphology of the stable transfected cell strains and CPE at 72 hpi; (B) Detection of the abundance of α-2,3 linked receptors on the stable transfected cell strains; (C~E) Detection of AIV proliferation effect by HA, $TCID_{50}$ and $EID_{50}$ assay. An asterisk(*) represented $p$ <0.05, two asterisks represented $p$ <0.01, and three asterisks represented $p$ <0.001. In Fig. 6, the culture conditions of the H9-4 isolate in different MDCK cell lines: the inoculation amount was $10^{-3}$ with the addition of 0.5 μg·mL$^{-1}$ TPCK-trypsin, the harvest time of AIV was at 96 hpi.

The results indicated that the fluorescence intensity of S8 cells were obviously improved, with higher content of α-2,3 linked receptors than those of the mock and S1 cells (Figure 6B). H9-4 was inoculated to S8 cells to measure the HA titer, $TCID_{50}$ and $EID_{50}$. Experimental results showed that the HA titer of H9-4 in S8 cells was significantly higher than control cells ($p<0.05$) (Figure 6C). $TCID_{50}$ and $EID_{50}$ were also higher, including very significant differences of $TCID_{50}$ between S8 cells and the control ($p<0.01$) (Figure 6D and E). Based on the above results, it concluded that S8 stable transfected cell strain created by transgenic technology could improve the cell surface α-2,3 lined receptors, the sensitivity and replication of the virus, thus it was more suitable for the proliferation of H9N2 subtype AIV. However, any physiological process in cells should often be the results of the combination of multiple genes. So the single gene transfer was also limited here. In order to increase yield of AIV in the cell culture, multiple approaches should be integrated together.

## CONCLUSION

For the cell culture of the virus in some specific cell lines, the obtaining of high-titer virus depends on many factors, including the properties of the virus itself, the sensitivity of the host cells, and the ideal conditions for the biosynthesis. In this study, two strategies improving cell culture conditions, reforming cell lines were used to improve the yield of the virus. The experimental results showed that during the culture of the H9-4 isolate in MDCK cell lines, when the inoculation amount was $10^{-3}$~$10^{-4}$ with the addition of 0.5 μg·mL$^{-1}$ TPCK-trypsin, the proliferation of AIV was excellent at 96~108 hpi. At different stages of AIV culture, the re-addition of 0.3 μg·mL$^{-1}$ TPCK-trypsin in the maintenance media could increase the titer of the virus to a certain extent. Also, the addition of 40 mmol·mL$^{-1}$ HEPES or 3 mmol·mL$^{-1}$ Gln in the maintenance media could improve the proliferation of AIV, while the addition of serum or BSA significantly inhibited the proliferation of the virus. Among them, trypsin was the key factors influencing the proliferation of H9N2 subtype AIV by optimizing the cell culture conditions. To further increase the sensitivity of H9N2 subtype AIV to the host cells, the chicken *St3gal* Ⅰ gene was cloned, and was transfected into MDCK cell lines. 1 strain of highly-expressed *St3gal* Ⅰ-positive monoclonal cell strain S8 was detected, of which the α-2,3 linked receptor abundance was evidently increased. After testing, it was found that the stably-expressed *St3gal* Ⅰ-positive cell strain S8 could improve the infection sensitivity and replication capacity of the isolates of H9N2 subtype AIV.

***Acknowledgements:*** *This study was funded by the Postdoctoral Fund of Shandong Binzhou Animal Science and Veterinary Medicine Academy Postdoctoral Research Station (BSH2014001), the Shandong Province Natural Science Fund Project (ZR2012CL14) and the Doctor Fund of Binzhou University (2010Y08).*

## REFERENCES

1. D.J. Alexander, *Avian Dis.*, **51**, 161 (2007).
2. N.J. Cox, K. Subbarao, *Annu. Rev. Med*., **51**, 407 (2000).
3. G.Y. Dong, J. Luo, H. Zhang, C. Wang, M. Duan, T.J. Deliberto, D.L. Nolte, G. Ji, H. He, *PLoS One*, **6**(2), e17212 (2011).
4. D.J. Alexander, *Avian Dis.*, **47**,792 (2003).
5. I.H. Brown, J. Banks, R.J. Manvell, S.C. Essen, W. Shell, M. Slomka, B. Londt, D.J. Alexander, *Dev. Biol. (Basel).*, **124**, 45 (2006).
6. S. Shahsavandi, M.M. Ebrahimi, A. Mohammadi, N. Zarrin Lebas, *Cytotechnology.*, **65**, 419 (2013).
7. Z.Q. Wu, J. Ji, K.J. Zuo, Q.M. Xie, H.M. Li, J. Liu, F. Chen, C.Y. Xue, J.Y. Ma, Y.Z. Bi, *Point Sci.*, **89**, 1136 (2010).
8. R. Parvin, A.A. Shehata, K. Heenemann, M. Gac, A. Rueckner, M.Y. Halami, T.W. Vahlenkamp, *Veterinary Research*., **46**, 75 (2015).
9. Y.P. Lin, M. Shaw, V. Gregory, K. Cameron, W. Lim, A. Klimov, K. Subbarao, Y. Guan, S. Krauss, K. Shortridge, R. Webster, N. Cox, A. Hay, *Proc. Natl. Acad. Sci. USA.*, **97**, 9654 (2000).
10. A. Fusaro, I. Monne, A. Salviato, V. Valastro, A. Schivo, N.M. Amarin, C. Gonzalez, M.M. Ismail, A.R. Al-Ankari, M.H. Al-Blowi, O.A. Khan, A.S. Maken Ali, A. Hedayati, G.J. Garcia, G.M. Ziay, A. Shoushtari, K.N. Al Qahtani, I. Capua, E.C. Holmes, G. Cattoli, *J. Virol.*, **85**, 8413 (2011).
11. Q.Y. Teng, W. Shen, Q. Liu, G. Rong, L. Chen, X. Li, H. Chen, J. Yang, Z. Li, *J. Virol*, **12**, 143 (2015).
12. A.S. Monto, H.F. Maassab, E.R. Bryan, J. *Clin Microbiol.*, **13**, 233 (1981).
13. J.S. Robertson, C.W. Naeve, R.G. Webster, J.S. Bootman, R. Newman, G.C. Schild, *Virology.*, **143**, 166 (1985).
14. E. Hoffmann, S. Krauss, D. Perez, R. Webby, R.G. Webster, *Vaccine.*, **20**, 3165 (2002).
15. D. Onions, W. Egan, R. Jarrett, D. Novicki, J.P. Gregersen, *Biologicals.*, **38**, 544 (2010).
16. J.P. Gregersen, H.J. Schmitt, H. Trusheim, M. Bröker, *Future Microbiol*., **6**, 143 (2011).
17. S.Z. Feng, P.R. Jiao, W.B. Qi, H.Y. Fan, M. Liao, *Appl. Microbiol Biotechnol*., **89**(4), 893 (2011).
18. N.A. Ilyushina, M.R. Ikizler, Y. Kawaoka, L.G. Rudenko, J.J. Treanor, K. Subbarao, P.F. Wright, *J. Virol.*, **86**, 11725 (2012).
19. V.Y. Lugovtsev, D. Melnyk, J.P. Weir, *PLoS One*., **8**(9), e75014 (2013).
20. J. Post, E.D. de Geus, L. Vervelde, J.B. Cornelissen, J.M. Rebel, *Virol J.*, **10**, 23 (2013).
21. K. Srinivasan, R. Raman, A. Jayaraman, K. Viswanathan, R. Sasisekharan, *PLoS One*., **8**, e59550 (2013).
22. S. Thongratsakul, Y. Suzuki, H. Hiramatsu, T. Sakpuaram, T. Sirinarumitr, C. Poolkhet, P. Moonjit, R. Yodsheewan, T. Songserm, *Asian Pac. J. Allergy Immunol*, **28**, 294 (2010).
23. X.Y. Li, J.Z. Shi, J. Guo, G. Deng, Q. Zhang, J. Wang, X. He, K. Wang, J. Chen, Y. Li, J. Fang, H. Kong, C. Gu, Y. Guan, Y. Suzuki, Y. Kawaoka, L. Liu, Y. Jiang, G. Tian, Y. Li, Z. Bu, H. Chen, *PLoS Pathog.*, **10**, e1004508 (2014).
24. H.A. Shaib, N. Cochet, T. Ribeiro, A.M. Abdel Nour, G. Nemer, E. Azhar, A. Iyer, T. Kumosani, S. Harakeh, E.K. Barbour, *J. Infect Dev. Ctries.*, **8**(5), 570 (2014).
25. T. Ito, Y. Kawaoka, Vet. Microbio1., 74, 71 (2000).
26. R.J. Russell, D.J. Stevens, L.F. Haire, S.J. Gamblin, J.J. Skehel, *Glycoconj J.*, **23**, 85 (2006).
27. S.D. Pawar, S.S. Parkhi, S.S. Koratkar, A.C. Mishra, *J. Virol*., **9**, 251 (2012).
28. I. Stephenson, J.M. Wood, K.G. Nicholson, M.C. Zambon, *J. Med. Virol*., **70**(3), 391 (2003).

29. A.S. Gambaryan, A.B. Tuzikov, G.V. Pazynina, R.G. Webster, M.N. Matrosovich, N.V. Bovin, *Virology.*, **326**(2), 310 (2004).
30. H.D. Klenk, R. Wagner, D. Heuer, T. Wolff, Virus Res., 82, 73 (2002).
31. M. Matrosovich, T. Matrosovich, J. Carr, N.A. Roberts, H.D. Klenk, *Virology.*, **77**, 8418 (2003).
32. S. Ge, Z. Wang, *Critical reviews in microbiology*., **37**, 157 (2011).
33. M. Yamoka, M. Homma, H. Hotta, *Arch. Virol*., **140**, 937 (1995).
34. J.Baron, C. Tarnow, D. Mayoli-Nüssle, E. Schilling, D. Meyer, M. Hammami, F. Schwalm, T. Steinmetzer, Y. Guan, W. Garten, H. D Klenk, E. Böttcher-Friebertshäuser, *J. Virol.*, 1811 (2013).
35. C.Y. Lu, J.H. Lu, W.Q. Chen, L.F. Jiang, B.Y. Tan, W.H. Ling, B.J. Zheng, H.Y. Sui, *Chin. Med. J.*, **121**(20), 2050 (2008).
36. S. Hatakeyama, Y. Sakai-Tagawa, M. Kiso, H. Goto, C. Kawakami, K. Mitamura, N. Sugaya, Y. Suzuki, Y. Kawaoka, *J. Clin. Microbiol.*, **43**(8), 4139 (2005).
37. X. Zheng, C.J. Cardona, J. Anunciacion,1 S. Adams, N. Dao, *Journal of General Virology*., **91**, 343 (2010).
38. L.J. Reed, H. Muench, *Am. J. Hyg*., **27**, 493 (1938).
39. Y. Genzel, U. Reichl,. *Expert Rev. Vaccines*., **8**(12), 1681 (2009).
40. J.M. Audsley, G.A. Tannock, *Drugs.*, **68**(11), 1483 (2008).
41. N.R. Hegde, *Hum. Vaccin Immunother*., **11**(5), 1223 (2015).
42. S.H. Seo, O. Goloubeva, R. Webby, R.G. Webster, *J. Virol*, **75**(19), 9517 (2001).
43. O. Kistner, N. Barrett, W. Mundt, M. Reiter, S. Schober-Bendixen, G. Eder, F. Dorner, *Vaccine*., **18**(1), 50 (2001).
44. G. Kemble, H. Greenberg, *Vaccine.*, **21**(16), 1789 (2003).
45. M. Luo, *Adv. Exp. Med. Biol*., **726**, 201 (2012).
46. T. Ito, Y. Suzuki, A. Takada, A. Kawamoto, K. Otsuki, H. Masuda, M. Yamada, T. Suzuki, H. Kida, Y. Kawaoka, *Virology.*, **71**(4), 3357 (1997); Y. Wang, J. Liu, J. Lan-Ying, Q. Zhou, X.Y. Zeng *Marine environmental science*, **32**, 456 (2013).

# ОПТИМИЗАЦИЯ НА КЛЕТЪЧНА КУЛТУРА НА H9N2 ПОДТИП НА AIV И УСТАНОВЯВАНЕ НА КЛЕТЪЧЕН ЩАМ С ВИСОК ДОБИВ

Ю-Джиан Лю[1,2,3], Ксин-Мин Ву[4], Ю Гуан[4], Сонг-Лин Жанг[4], Джин-Лиан Ван[4], Джиу-Ксиа Жао[5], Вен-Ю Хан[2], Жи-Цян Шен[1,4,5] *

*[1]Постдокторска програма, [4] Академия за животновъдни науки и ветеринарна медицина Шандун Бинчжоу, Бинчжоу, Китай*
*[2] Постдокторска програма, Дзилин университет, Чанчун, Китай.*
*[3]Отделение на науките за живота, Университет в Бинчжоу, Бинчжоу, Китай.*
*[5] Шандунски институт по биотехнология, Бинчжоу, Китай. Бинчжоу, Китай.*

Получена на 10 октомври 2016 г.; ревизирана на 10 февруари 2016 г.

(Резюме)

Тенденцията в производството на противогрипна ваксина е полечаването и от клетъчни култури. Това проучване имаше за цел да установи ключовите технологии на клетъчната култура на подтип H9N2 на AIV (вируса на птичия грип). Резултатите показват, че по време на култивиране на клетъчни линии от изолат H9-4 в MDCK (Мадин-Дарби кучешки бъбрек), когато инокуломът е $10^{-3}$ ~ $10^{-4}$ с 0.5 мкг мл$^{-1}$ TPCK (тозил фенилаланил хлорметил кетон) -трипсин, 40 ммол мл$^{-1}$ HEPES (хидроксиетилпиперазин етансулфонова киселина) или 3 ммол ·мл$^{-1}$ Gln (глутамин) в средата за поддръжка, разпространението на AIV беше отлично в 96 ~ 108 HPI (час след заразяването). Освен това, добавката на 0.3 мкг мл$^{-1}$ TPCK-трипсин може да увеличи титъра на вируса до известна степен. Пилешки ген St3galI бе клониран и трансфектиран в клетъчни линии MDCK. Чрез скрининг, бе получен един щам на моноклонален клетъчен щам S8, със силно изразена продукция на *St3galI*, от които излишъка на α -2,3 свързан рецептор очевидно се увеличава. Значително се увеличава разпространението на клетки S8 на H9-4. Резултатите потвърдиха, че инфекционната чувствителност и капацитетът на репликация на изолатите на H9N2 подтип на AIV бяха допълнително подобрени чрез увеличаване на излишъка на рецептор на повърхността на клетката гостоприемник.