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Optimization of Equine Influenza Strain A/equine/Otar/764/07 (H3N8) Cultivation in MDCK Cell Culture

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ABSTRACT

The paper presents the results of a study of cultural characteristics of the strain A/equine/Otar/764/07 (H3N8) of equine influenza virus isolated from sick horse during the outbreak of equine influenza epizooty in the Republic of Kazakhstan. The studies determined the optimal culture conditions for equine influenza virus strain A/equine/Otar/764/07 (H3N8) in MDCK cell culture. The optimal infecting dose of the virus was 0,1 TCE_{50}/cell, temperature and cultivation duration - 34 ± 0,5 ° C, 72 h, respectively, and Trypsin concentration in medium - 2 mg / ml. Subject to the determined parameters of A/equine/Otar/764/07(H3N8) strain cultivation in MDCK the virus-containing suspension with an infectious titer of at least 7,00 lg TCE_{50}/ml and hemagglutinating titer of 8,0 $\log_2$ can be obtained. The results obtained during the strain reproductive characteristics study can be used for the development of vaccines and diagnostic kits.

KEYWORDS: virus, equine influenza, cultivation, cell culture.

INTRODUCTION

Equine influenza is an infectious, acute, highly contagious disease of horses, which is spread throughout the world and characterized by short-term fever, general depression, conjunctivitis, catarrhal inflammation of the upper respiratory tract, dry, jerky, deep and painful cough, in severe cases, pneumonia [1 - 3].

The first mention of equine influenza cases relates to 1688 (England and Ireland).

In 1775-1776 outbreaks of equine influenza were registered on the European continent.

Currently, the disease is widespread and is registered in Europe, the U.S., Russia, China, Brazil, Azerbaijan, Iran, Mongolia, Australia, Japan and some other countries [1, 4].

In the Republic of Kazakhstan epizooty of equine influenza was first registered in May 1992. It started in the farms of Western Kazakhstan, Aktobe, Atyrau, South Kazakhstan and Zhambyl region. The last outbreak of equine influenza was spread in the east (East Kazakhstan, Semipalatinsk), central (Karaganda) and southern (Kyzylorda, South Kazakhstan and Zhambyl) regions [5].

Due to the disease wide spread the international community worked out the following recommendations in key control areas against equine influenza: ensuring biological safety, epidemiology / epizootiology, diagnostic and vaccination measures, preparation of legislation and public information.

To study the equine influenza virus (EIV) and the development of diagnostics and prophylactic means it is necessary to get a highly active virus containing material, which can be achieved through the selection of the optimal bio-system and parameters of virus cultivation. The virus cultivation takes a special place in the technology of influenza vaccines production. In 1968 Gaush and Smith reported the equine influenza virus replication and plaque formation in the continuous dog kidney cell line (MDCK). Application of the method of influenza viruses' isolation in cell culture increases the likelihood of rapid isolation and identification of a new influenza strain that is of particular importance in the pre-pandemic period. [6]. In recent years, the literature references contain new data indicating that influenza vaccines prepared on MDCK cells protect experimental animals better than their embryonic analogs [7]. It is shown that immunization of different animal species with either culture or embryonic influenza vaccines induced the same level of antihemagglutination antibody production [8].

The aim of this work was to study the cultural characteristics and to determine the optimal parameters of EIV A/equine/Otar/764/07 (H3N8) strain cultivation in MDCK cell culture to obtain a highly active virus-containing material suitable for the development of prophylactic and diagnostic means.

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MATERIALS AND METHODS

Virus

EIV A/equine/Otar/764/07 (H3N8) strain isolated from sick horse in the Republic of Kazakhstan in 2007 by RIBSP scientists, with the infectious titer of 8,45 ± 0,14 lg EID_{50}/ml and haemagglutination reaction titer 10 log_2 was used in the experiments.

Cell culture and medium

A continuous MDCK cell line grown in minimum Dulbecco's modified IGLA medium (DMEM), containing 10% fetal calf serum, 1% essential amino acids, penicillin (100 U/ ml), streptomycin (100 µg / ml), and Fungizone (1 , 0 g / ml) was used in the studies.

Additives

Lyophilized trypsin (Trypsin, from bovine pancreas) (Sigma-Aldrich Chemie GmbH, USA) was diluted in Hanks balanced salt solution to prepare a 1% stock solution and stored at -20 ˚ C.

Virus cultivation

Cultivation of EIV A/equine/Otar/764/07 (H3N8) strain was implemented in MDCK cell culture. Cell culture was infected with the dose of 0,1 TCE_{50}/cell followed by incubation at 34 ± 0,5 ° C, for 72 hours, with daily examination under a light microscope. When the destruction of the monolayer achieved at least 70-80% vessels with the infected cell culture were frozen at -70 ° C for 12-14 hours.

For elaborating the EIV A/equine/Otar/764/07 (H3N8) strain cultivation conditions we conducted a study to determine the optimal incubation temperature of EIV. The virus-infected cell cultures were incubated at 32 ° C, 34 ° C and 37 ° C. Also we conducted studies to determine the virus accumulation level with different infecting doses 0.0001, 0.001, 0,01 and 0.1 TCE_{50}/cell. For determining the terms of cultivation we cultured the virus for 48, 72, and 96 hours, after a specified time some part of culture containing vessels were frozen at -70 ° C for 12-14 hours. Samples from each vessel with virus-containing suspension were taken to determine the infectious titer in quantitative hemagglutintion reaction.

Haemagglutination reaction

For the haemagglutination reaction two-fold dilutions of virus-containing material in the 0.87% solution of sodium chloride were prepared the virus was titrated in 96-well U-shaped microplates. 0.5% suspension of cock erythrocytes was added to all wells with virus dilutions. Result of the reaction was evaluated visually in crosses (one to four) on erythrocyte agglutination. The virus hemagglutinating activity titer was considered the highest dilution in which agglutination of red blood cells was observed by at least two crosses.

Determination of virus infectivity in cell culture

Activity was determined by titration of the virus in cell culture. 10-fold dilutions of virus containing suspension from 10^{-1} to 10^{-8} were prepared in DMEM medium. Accounting of the titration results was conducted by the presence of cytopathic changes in tubes with infected monolayer during its absence in the control tubes [9].

The virus titer was expressed as lg TCE_{50}/sm^3, which was calculated by the Reed and Mench method [10].

RESULTS

The studies to determine the EIV optimal incubation temperature were conducted. The studies of the incubation temperature effect on the EIV accumulation were performed in triplicate, followed by determination of haemagglutinating and infectious titer of virus-containing material. Results are presented in Figure 1.

From the data presented in Figure 1 it is seen that the virus reproduction was observed at all tested temperatures. The optimal incubation temperature was 34°C, where the infectious titer was 7,25 ± 0,12 lg TCD_{50}/ml, and the haemagglutination reaction titre was 8,0 log_2. Increasing or decreasing of culture temperature caused a 1-2 times decrease of virus infectious activity titer.

In the following experiments we determined the EIV A/equine/Otar/764/07 (H3N8) strain accumulation level at various doses of infection. Doses from 0,0001 to 0,1 TCE_{50}/cell were used under identical culture conditions. The results of these studies are shown in Figure 2.

The results of the studies (Figure 2) showed that accumulation of the virus in titers from 6,83 to 7,41 lg TCD_{50}/ml and haemagglutinating titer from 7.0 to 8,0 log_2 respectively was observed when using infecting dose from 0.01 to 0.1 TCE_{50}/cell. Using virus infection doses less than 0.01 TCE_{50}/cell for producing virus-containing material in cell culture is not appropriate because the level of accumulation of infectious and hemagglutinating activity of the virus was significantly lower. Thus, the optimal infection dose for EIV A/equine/Otar/764/07 (H3N8) strain in MDCK cell culture is 0.1 TCE_{50}/cell.

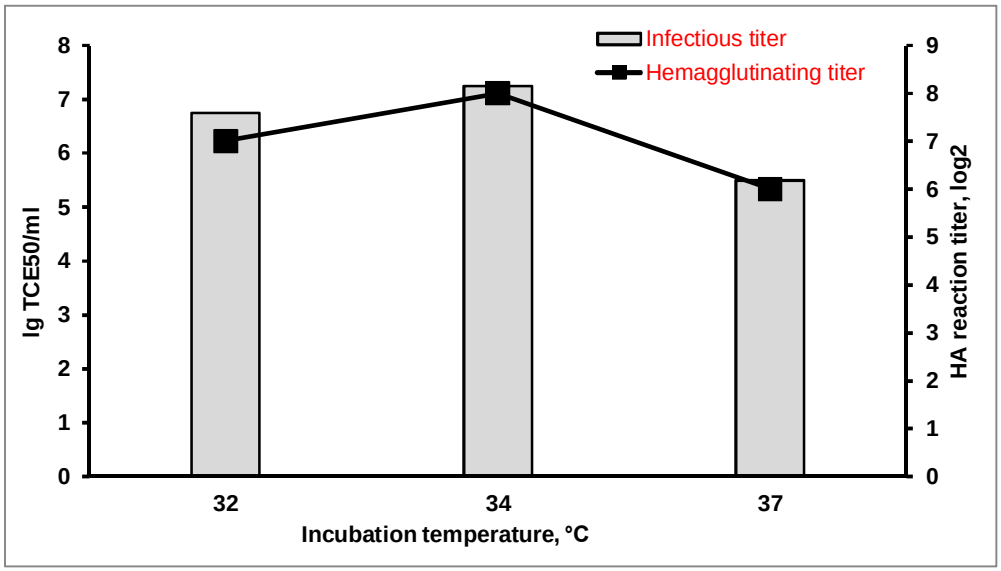
To determine the period of cultivation of A/equine/Otar/764/07 (H3N8) strain in MDCK cell culture the cells' monolayer was infected with a dose of 0.1 TCD_{50} followed by incubation at 34°C. The research results are presented in Figure 3.

The data at Figure 3 show that EIV A/equine/Otar/764/07 (H3N8) strain is accumulated in the largest titers after 72 hours, wherein the viral titer in haemagglutination reaction is 8,0 log_2, and infectious virus titre is 7,25 ± 0 08 lg TCD_{50}/ml. When incubation lasted for 48 hours infectious and hemagglutinating activity was significantly lower. The increase of virus cultivation time up to 96 hours resulted in a decrease of both infectious and haemagglutinating titer.

In the next series of experiments we conducted studies to determine the effectiveness of proteases in the culture medium. The enzyme Trypsin at various concentrations was used for hemagglutinin proteolysis. The results are shown in Table 1.

Having evaluated the nature of virus cytopathic effect manifestation and the area of cell monolayer lesions it can be concluded that the most optimal concentration of trypsin in the medium was 2 µg/ml. The virus cytopathic effect has been observed for 18-40 hours, which is presented in Figure 4.

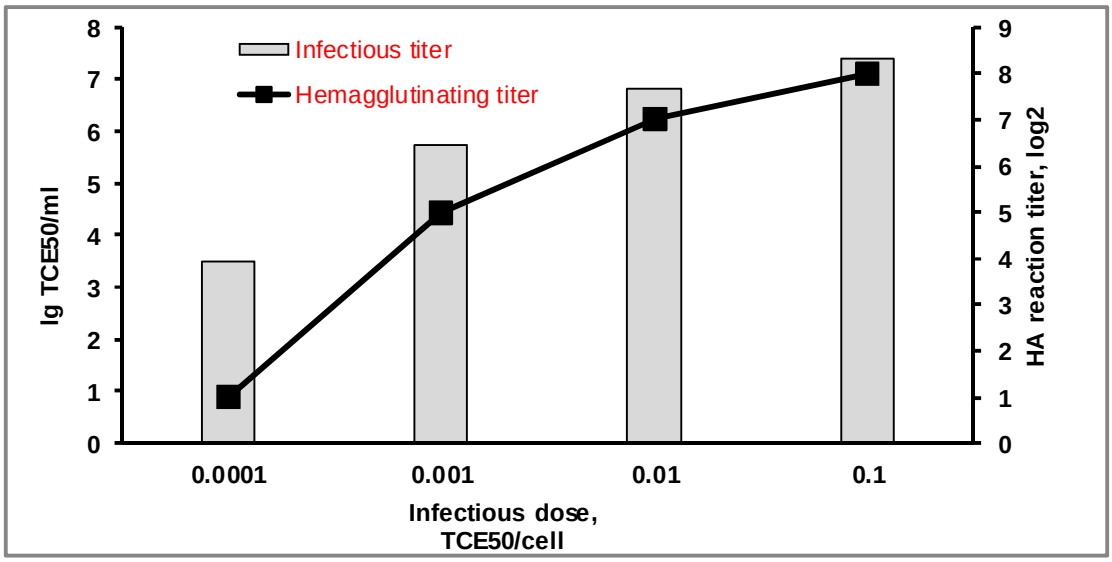
Thus, it was found that Trypsin, from bovine pancreas at a concentration of 2 mg/ml promotes the most efficient reproduction of EIV in MDCK cell culture, and the virus accumulation with infective activity 7,08 ± 0,08 lg TCE_{50}/ml and hemagglutinating titer 8,0 log_2 was observed.



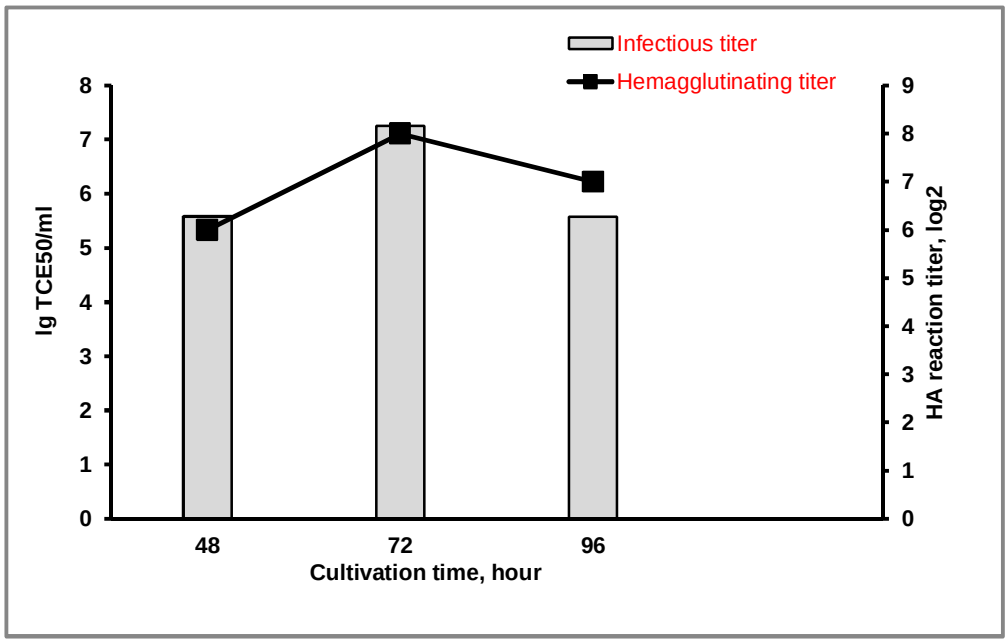
Picture 1 – Level of EIV A/equine/Otar/764/07 (H3N8) strain accumulation in MDCK cell culture depending on incubation temperature

Table 1 – Reproduction of EIV A/equine/Otar/764/07 (H3N8) strain in MDCK cell culture with proteolytic hemagglutinin activation by tripsin

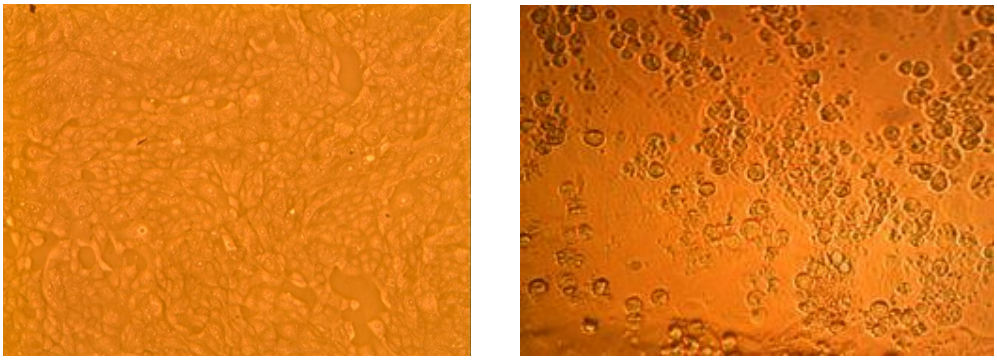
Protease	Enzyme concentration	Infectious activity lg TCE_{50}/ml (x ± m), n=3	HA reaction titer, log_2
Trypsin, from bovine pancreas SIGMA	2 µg/ml	7,08 ± 0,08	8,0
	1,5 µg/ml	6,08 ± 0,16	7,0
	1 µg/ml	5,50 ± 0,14	6,0
	0,5 µg/ml	4,75 ± 0,14	4,0
Control	-	-	-
Note «-» - negative result			



Picture 2 – Level of EIV A/equine/Otar/764/07 (H3N8) strain accumulation in MDCK cell culture depending on infectious dose



Picture 3 – Level of EIV A/equine/Otar/764/07 (H3N8) strain accumulation in MDCK cell culture depending on cultivation terms



Non-infected cell culture CPE of A/equine/Otar/764/07 strain

Picture 4 – Light microscopy of MDCK cell culture before and after infection with EIV, magnification 9×0,20

DISCUSSION

Horses, like other farm animals have various diseases, among which contagious diseases play special role. In the last few years a viral infection commonly known as the "horse flu" has become especially important for horse breeding [11].

MDCK cells are optimal cell system for the isolation and cultivation of influenza virus. In recent years, in the literature references there are new data indicating that influenza vaccines produced on MDCK cells protect experimental animals better than their embryonic analogs [7].

According to the references MDCK cell culture is the most sensitive cell line for the reproduction of influenza viruses, and it is recommended by the World Health Organization for the isolation of influenza viruses [12].

The increase of virus production is of great practical interest, particularly in the mass production of virus preparations. Many research works are dedicated to the study of various factors influence on the virus reproduction and the accumulation of viral antigens in cell culture.

The success of virus cultivation primarily depends on the right choice of the cell substrate. The main criteria for this are the high yield of virus and viral antigen, the relative simplicity to the culture conditions and the safety of the vaccine. Some viruses replicate easily in cell cultures of foreign origin, others - prefer natural host cells or differentiated, or require specific culture conditions (reduced temperature, trypsin addition, high or low multiplicity of infection), and others - in general have not been able to replicate outside a living organism [13, 14].

During the cultivation of viruses in cell culture, its activity depends on several conditions such as temperature of incubation, infecting dose and cultivation time terms [15].

Viruses replicate in sensitive cells in a specific temperature zone, beyond which the mature viral particle formation does not occur. Each virus has its own genetically conditioned optimum temperature which cause replication [16].

Temperature limits and a temperature optimum of virus replication is controlled by the viral genome, although to a certain extent, it also depends on the cell system. It is believed that the optimum temperature for the reproduction of most viruses is 36 - 37 ° C, but this rule has exceptions. For example, influenza C virus, in contrast to influenza A and B, replicates better at 32 - 33°C [17]. We found that the optimal temperature for incubation of EIV A/equine/Otar/764/07 strain is 34 ± 0,5°C for 72 hours. In our opinion, the increase of cultivation time causes partial inactivation of the EIV, which negatively affects the pathogen activity. Our results are consistent with those of N.P. Shmelev et.al., who say that the standard procedure of influenza virus isolation in MDCK culture allows diagnosing the flu not earlier than 72 hours [18].

The virus accumulation in cell culture also depends on infection dose multiplicity, which mainly affects the duration of virus accumulation and not on its harvest. Usually the dose 0,001-0,1 TCE_{50} per cell is applied for monolayer cultures infection and virus accumulation. In this case, the virus accumulates due to the many cycles of reproduction. The multiplicity of infection is more important when viability of a cell culture reduces rapidly, and viruses do not have a short cycle of reproduction [17, 19]. In the course of our studies we determined that the optimal infective dose is 0,1 TCE_{50} per cell.

Proteolytic processing of virion polyproteins occurs in viruses of many families. Virus specific and cellular proteases take part in proteolytic processing. As protease cells participate in proteins processing in viruses it can be assumed that all viruses should increase the reproduction in the presence of trypsin in the growth medium. The degree of enhancement effect in this case will be in inverse proportion to the proteolytic

activity of intracellular enzymes of the culture system. Studies of influenza virus cultivation in animal cells indicate that the introduction of proteases (usually trypsin) in the culture medium is necessary for the formation of a high grade of infectious virus with high accumulation of hemagglutinating antigen and its ability to reproduction. [20] In this regard, we studied the effect of proteolytic enzymes on the equine influenza virus replication in cell culture. Trypsin, from bovine pancreas (SIGMA) was used as the proteolytic enzyme. Studies have shown that the most optimal concentration of trypsin in the medium is 2 μg / ml.

Thus, following the determined parameters of EIV cultivation in MDCK cell culture it is possible to obtain a highly active virus-containing material for the development of prophylactic and diagnostic means.

Acknowledgment

The authors declare that they have no conflicts of interest in the research.

REFERENCES

1 Syurin, V.N., Belousova, R.V., Fomina, N.V., 1991. Directory: Diagnosis of viral diseases of animals. Moscow, Agropromizdat, pp: 178 -182.

2 Foord, A.J., P Selleck, A Colling, J Klippel, D Middleton, H.G. Heine, 2009. Realtime RT-PCR for detection of equine influenza and evaluation using samples from horses infected with A/equine/Sydney/2007 (H3N8). Vet Microbiol. May 28;137(l-2): l-9.

3 Janet M. Daly, J. Richard Newton, Jennifer A. Mumford, 2004. Current perspectives on control of equine influenza. Vet.Res. pp: 411–423

4 Information and Analysis Center of Rosselkhoznadzor FGI "Federal Centre for Animal Health", 2007. Summary of the epizootic situation with especially dangerous animal diseases in the countries of Eastern and South-East Asia and Oceania.

5 Rozamova R.A., R.U. Beisembayeva, L.M. Fursova, T. Glebova, Z.K. Chuvakova, 1994 Epizooty of equine influenza in Kazakhstan in 1992. Scie. Conf. Proc. "Actual problems of Virology (molecular biology, immunology, diognostics, biotechnology, epidemiology and epizootiology)" 18 - 20 May, Gvardeiskiy, Kazakhstan, Part I. - P.120.

6 Instruction # 30, 10.03.2006 "Organization of AI preventive and anti-epidemic measures". Resolution of Chief State Sanitary Vet of the Republic of Belarus.

7 Gorbunov A., T.V. Pysina, 1973. Influenza in animals. M., "Kolos", pp: 26-31.

8 http://lib.bioinfo.pl/pmid:20307595

9 Ján Szántó, January 1956. Titrácia vírusu chrípky v tkanivových kultúrach. Československá Mikrobiologie, Volume 1, Issue 1, pp: 41-46.

10 Syurin V.N., R.V. Belousova, N.V. Fomina, 1973. Veterinary Virology. M., "Kolos", pp: 267-272.

11 Bessarabov B.F., A.A.Vashu, E.S.Voronin et.al., 2007. Infectious diseases of animals. M: "Kolos", pp: 408-410.

12 Gert van Zyl Laboratory Findings, 2006. Influenza Report. Chapter 7. pp: 150–159.

13 Peyliza, P., D.U. Kingsbury, 1986. Genetics of influenza viruses. M., Medicine.

14 Guo, X., M. Liao, C. Xin 2003. Sequence of HA gene of avian influenza A/Chicken/Guandong/SS/1994(H9N2) virus. Avian diseases, 47, 1118-1121.

15 Ershebulov Z.D., K.D. Zhugunisov, D.S. Taranov, E.O. Abduraimov, et al., 2009. The cultivation of the influenza virus strain “NIBRG-14” in MDCK cell culture. International Scie. Conf. Mat.” New generation of influenza vaccines” P. 81

16 Lab M., Kirn A.C., 1968. r. Acad. ski., 268, 2624

17 Savelyev I.V., 1998. General physics. Moscow: Nauka, 4.

18 Shmelev NP, Gribkova N.V., 2009. New approaches in the laboratory diagnosis of influenza. GE RI of Epidemiology and Microbiology, Ministry of Health of the Republic of Belarus, Minsk, # 1.

19 Sergeev V.A., 1976. Reproduction and cultivation of animal viruses. M., "Kolos", P. 230.

20 Suares, D.L., 1998. Multiple alignment comparison of the non-structural genes of influenza A viruses. M.L. Perdue. Virus Research, 54, pp: 59-69.