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RESEARCH ARTICLE

The Method Evaluation of Culturing DF-1 to Proliferate Canine Distemper Virus in Mink with Cephodex Microcarrier

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Abstract: As an acute and highly lethal infectious disease, there is no specific therapeutic drug for canine distemper (CD). Although the process of large-scale production of canine distemper virus (CDV) vaccine of mink has been greatly improved, there are still many deficiencies to be perfected. As one of the most promising technologies for large-scale vaccine production, microcarrier suspension culture technology needs to be further improved. In this study, the application effect of the new Cephodex microcarrier in CDV culture was evaluated to establish a set of technical process for DF-1 cell high-density growth and CDV efficient proliferation. To perfect the large-scale CDV production process, Cephodex was used to suspension culture DF-1 cells for proliferating CDV. In a shake flasks culture system, the optimal culture conditions were established by optimizing culture temperature, virus inoculation and harvest time. Therefore, mink CD vaccine high-efficiency production was laid on the preliminarily established technology of CDV microcarrier suspension culture. The cell density could reach over 3×10^6 cells/mL after 72 h cultured with Cephodex microcarrier at 37°C. Proliferated at 35°C, the CDV titer after 72 h was about $10^{0.5}$ $TCID_{50}$/0.1mL higher than that at 33°C and 37°C. These results show that the Cephodex microcarrier could be used for large-scale culture of DF-1 cells and efficient proliferation of CDV.

Keywords: *Canine distemper virus, DF-1 cells, Microcarrier, Suspension culture, Vaccine*

Minklerde Canine Distemper Virüsünün Cephodex Mikro Taşıyıcı İle Çoğaltılması İçin DF-1 Kültür Yönteminin Değerlendirmesi

Öz: Akut ve oldukça ölümcül bir enfeksiyöz hastalık olan köpek distemperi (CD) için spesifik bir terapötik ilaç yoktur. Minklerde canine distemper virüsü (CDV) aşısının kitlesel üretim süreci büyük ölçüde geliştirilmiş olsa da, halen iyileştirilmesi gereken birçok eksiklikleri vardır. Büyük ölçekli aşı üretimi için en umut verici teknolojilerden biri olan mikro taşıyıcı sıvı kültür teknolojisinin daha da geliştirilmesi gerekmektedir. Bu çalışmada, yüksek yoğunluklu DF-1 hücre eldesi ve CDV'nin daha verimli üremesi için bir dizi teknik süreç oluşturmak amacıyla yeni Cephodex mikro taşıyıcısının etkinliği değerlendirildi. Büyük ölçekli CDV üretim sürecinin iyileştirilmesi amacıyla, CDV'yi üretmede kullanılan DF-1 hücre kültürünü sıvı hale getirmek için Cephodex kullanıldı. Çalkalamalı kültür şişeleri sisteminde, kültür sıcaklığı, virüs inokülasyonu ve toplama zamanı optimize edilerek optimal kültür koşulları oluşturuldu. Böylelikle, önceden kurulmuş CDV mikro taşıyıcı sıvı kültürü teknolojisinin üzerine yüksek verimlilikte mink CD aşısı üretimi gerçekleştirildi. Hücre yoğunluğu, Cephodex mikro taşıyıcı ile 37°C' de kültüre edildikten sonra 72 saat sonunda 3 x 10^6 hücre/mL' nin üzerine çıktı. 35°C'de üretilen CDV titresi, 72 saat sonra 33°C ve 37°C' dekinden yaklaşık $10^{0.5}$ $TCID_{50}$/0.1 mL daha yüksekti. Bu sonuçlar, Cephodex mikro taşıyıcısının, büyük ölçekli DF-1 hücre kültürü ve CDV'nin verimli bir şekilde üretilmesi için kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Canine distemper virus, DF-1 hücresi, Mikro taşıyıcı, Sıvı kültür, Aşı*

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Introduction

Canine distemper (CD) is an acute and highly lethal infectious disease of canines, which causes great losses to the global fur economic animal breeding every year [1]. China is a large fur animal breeding country, which is also greatly affected by this disease [2]. Since the first report on the prevalence of mink CD in China, it has spread to Chinese major fur animal breeding areas after half a century, such as Hebei, Shandong, Heilongjiang. Especially in recent years, fur animals have developed rapidly with the breeding concentrated & huge scale and the fur animal active market, which have created favorable conditions for the prevalence of this disease [3]. At present, there is no specific therapeutic drug for the disease, and immunization is still an effective mean of prevention and control [4,5]. Therefore, high-quality vaccine products are particularly important. Usually, virus antigen is the core factor affecting the quality of vaccine. The proliferation level of canine distemper virus (CDV) on cells is generally not high, which affects the immunogenicity of antigen in vaccine and leads to clinical immune failure [6-8].

For a long time, the traditional two dimensional (2D) cell culture technology was mostly used to proliferate CDV, but the efficiency was low with low viral titer. So it was difficult to have an ideal application effect in large-scale production. Afterwards, some methods were performed such as screening high-quality cell lines and optimizing the culture process to improve the CDV proliferation efficiency and the viral titer, and some results had been achieved certainly [9,10]. Yang et al.[11] constructed a Vero/dSLAM cell line with the gene expressing dog signal lymphocyte activating molecule (dSLAM), and successfully cultured CDV (CD1901 strain) in the cells. The results showed that the highest viral titer of CD1901 could reach $10^{5.5}$ 50% tissue culture infectious dose ($TCID_{50}$)/mL in the Vero/dSLAM cells at the 4 day post inoculation. Because microcarrier culture technology has the characteristics of the three dimensional (3D) cell culture technology, it can effectively improve cell density and production efficiency. In recent years, this technology has been widely used in large-scale animal cell culture and industrialized vaccine production [12]. Saykally et al.[13] used microcarriers to culture ARPE-19 cells for human cytomegalovirus (HCMV) proliferation in bioreactor. The virus yield was 100 times that of the traditional culture plate method. At the same time, the treatment time was shortened by 90% compared with the traditional culture method, which greatly improved the work efficiency. Liu et al.[14] discovered that Cephodex microcarrier was very suitable for large-scale culture of CIK cells and the grass carp reovirus (GCRV) titer could reach the $10^{8.5}$ $TCID_{50}$/ml in this way, thus a solid basis could be established for the large-scale preparation of vaccine against the grass carp hemorrhage. In this study, the screened DF-1 cell line was cultured in high density with a new Cephodex microcarrier, and the technical conditions such as culture temperature were optimized to achieve the purpose of CDV efficient proliferation.

Material and Methods

Ethical Approval

The study was approved by the local Ethics Committee of Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou, China (Approval no: SDBZASVM-2020-002).

Reagents

Cell growth medium was DMEM (10-203, Wuxi Meidi biological products company) solution with 6% (v/v) FBS (11011-8611, Zhejiang Tianhang Biotechnology Co., Ltd), and cell maintenance medium was DMEM solution with 2% (v/v) FBS.

Cell Resuscitation and Subculture

One cryopreservation tube containing DF-1 seed cells (ATCC CRL-12203, Shandong Binzhou Animal Science and Veterinary Medicine Academy) was put into 37°C water bath, and these cells in which were thawed quickly and centrifuged at 1000 rpm at room temperature for 5 min, and then the supernatant was discard. After the cells were resuspended with cell growth medium, them were transfered into T75 flask aseptically. The flask was added with an appropriate amount of growth medium and put into incubator with 37°C and 5% CO_2. At 72 h, DF-1 cells grew into intact monolayer were digested by trypsin and dispersed by blowing, and then passaged with the expanded ratio of 1:3.

Microcarrier Preparation and Suspension Culture

Six 0.1 g Cephodex microcarriers (MC10001, Binzhou Biocarrier Biotechnology Co., Ltd) were put into six 50 mL conical shake flasks respectively, and 10ml PBS was added to each flask, and then the microcarrier was stirred and suspended with a glass rod. Six shake flasks were sterilized at 121°C for 30 min. After sterilization, the microcarrier was suspended when the temperature decreased to about 80°C to prevent microcarrier agglomerating. Replaced the sterile PBS, the shake flasks were injected 1~2 mL of cell growth solution, and then were put into incubate at 37°C for 24~72 h for sterility test.

Passing the sterility test, the supernatant was discarded and the microcarrier was retained for standby. DF-1 cells in T75 flask were treated with trypsin-EDTA digestive solution, blown to dispersing, collected aseptically, and the cell concentration was measured by trypan blue staining method [15,16]. 3×10^6 cells per flask were inoculated, and

Table 1. The shaker control parameters at different culture stage

Culture Stage	Temperature (°)	CO_2 (%)	Shaker Speed (rpm)	Shaker On/Off (min)
Intermittent shaking	37	5	55	3x(1/30) 4x(1/60)
Continuous shaking	37	5	60	ON

then the volume of cell growth medium was fixed to 10ml. These cells in every flask were mixed with microcarrier and cultured in minitron culture shaker (CH-4103 BOTTMINGEN, Infors, Switzerland). 0~6 h after cell inoculation is intermittent shaking, the intermittent time is 30~60 min, and the shaking time is 1~2 min *(Table 1)*. Then, DF-1 cells were cultured by microcarrier suspension in the continuous shaking culture stage [17,18]. Compared with intermittent shaking culture, the shaker parameter was set with some change in continuous shaking culture stage.

CDV Proliferation and $TCID_{50}$ Determination

After suspension culture for 72 h, DF-1 grew into intact cell monolayer. Samples were taken to observe the morphology of cell microcarrier complex. The cell culture medium in three shake flasks of the exposed group were replaced with cell maintenance medium, and CDV (provided by the Institute of Special Animal and Plant Sciences of CAAS, China) was inoculated according to 2% (v/v) [19]. As healthy cell control, the other three shake flasks for cells count were also replaced with cell maintenance solution. After infected and healthy cells were grouped, they were cultured at 33°C, 35°C and 37°C. Compared with continuous shaking culture of DF-1 cells, other shaker parameters were unchanged except for the culture temperature at the CDV proliferation stage. Samples were taken every 24 h after virus inoculation, and $TCID_{50}$ of supernatants were measured every 24 h according to Reed-Muench method.

Cell Density and Glucose Consumption

In the suspension culture stage, three shake flask cells were taken for cell counting every 24 h, and the glucose (G8270, Sigma-Aldrich) content in the culture medium was measured by biosensor analyzer (SBA-40E, Shandong Academy of Sciences). The other three bottles of cells for CDV proliferation later were only measured the glucose content without cell counting. The initial concentration of glucose in the culture medium of 6 shake flasks was 4.0 g/L. When the glucose content in the culture medium was lower than 2.0 g/L, 100 μL solution contained 200 g/L glucose was added to the culture medium in time to meet the nutritional needs of high-density cell growth [20-22].

According to the initial concentration of glucose in the culture medium, the amount of glucose added to the culture medium and the glucose content in the culture medium measured every 24 h, the daily glucose consumption of cells was calculated, and the changes of daily glucose consumption of cells before and after exposure were observed [23].

Statistical Analysis

All statistical analyses were performed using statistical package SPSS (version 22.0 for Windows, IBM SPSS Statistics) and $P<0.05$ was considered statistically significant.

Results

Morphology of Cells on Cephodex in Growth Stage

DF-1 cell suspension prepared by trypsin digestion and dispersion was evenly mixed with Cephodex microcarrier. After 6 h intermittent shaking culture, the cells could adhere to the microcarriers. At the time, most of these cells on the surface of microcarriers not spread are round, and a few spread cells are spindle *(Fig. 1-A)*. The DF-1 cells growth was observed on the surface of each microsphere, and there were almost no microbeads without cells to culturing 24 h *(Fig. 1-B)*. After 48 h, the cells monolayer had formed on more than 80% the microspheres surface *(Fig. 1-C)*. At 37°C, due to the rapid growth of DF-1 cells, a complete cell monolayer was formed on the surface of microbeads at 72 h [24]. On the surface of some microspheres, The cells further grew into a dense multi-layer structure, and then formed an obvious protrusion *(Fig. 1-D)*.

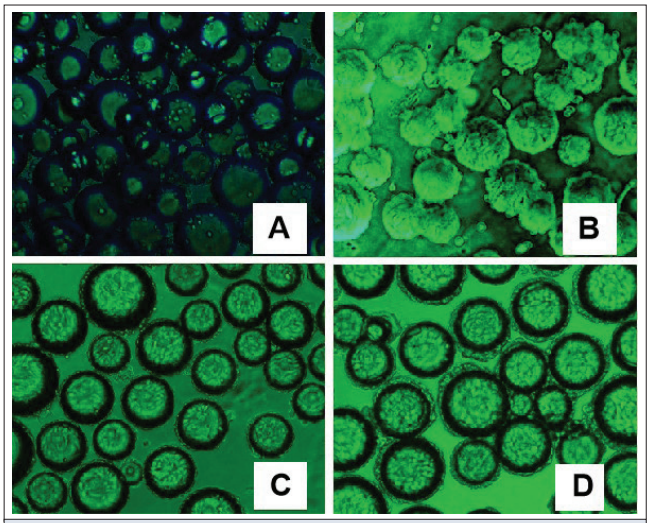

Fig 1. DF-1 cells on Cephodex in the different time at the growth culture stage. **A**- 6 h after cells inoculation; **B**- culture continuously for 24 h after cells inoculation; **C**- 48 h after cells grow; **D**- cells cultured for 72 h with Cephodex microcarrier

Growth Curve and Glucose Consumption in Growth Stage

For three flasks of DF-1 cells on microcarriers cultured at 37°C for 72 h, from the beginning of cell inoculation, samples was taken every 24 h for cell counting to determine cell density. At the same time, through the supernatant of all 6 flasks taken every 24 h, the glucose contents in the culture medium were measured to calculate the cell glucose consumption.

On the condition of 37°C, the cells in the three cells counted flasks grew rapidly, and the cell density increased rapidly too, reaching over 2.0×10^6 cells/mL at 48 h *(Fig. 2)*, and the cell glucose consumption also showed a rapid upward trend during this time *(Fig. 3)*. After 48 h, the cell density still increased, but the growth rate slowed down significantly. At 72 h, the cell density reached more than 3.0×10^6 cells/mL *(Fig. 2)*, and virus inoculation should be carried out at this time [25,26]. The cell growth curve and glucose consumption curve in this culture process showed that there was little difference in the cell growth curves among the three cells counted shake flasks ($P>0.05$), and their glucose consumption curves were also similar ($P>0.05$). Therefore, there were a high positive correlation between cell glucose consumption and cell density. In order to reduce the impact on the subsequent CDV proliferation experiment, the other three flasks of cells were not sampled for cell counting, but measured the glucose consumption. From the six flasks of cells glucose consumption curve, the cell density was very close to that of these cells counted flasks ($P>0.05$) *(Fig. 3)*.

The CPE of CDV Proliferation

Cytopathic effect (CPE) of DF-1 infected by CDV began to appear at 48 h post CDV inoculation, and a large number of cells shed from the surface of microcarriers. The cytopathy at different temperatures was observed. The cytopathy at 33°C was relatively light and the cell abscission was less *(Fig. 4-B)*. The lesion at 37°C was the most serious and the cell abscission was the most *(Fig. 4-D)*. The cytopathy and abscission at 35°C were between the two temperatures *(Fig. 4-C)*. At this time, there were still a large number of cells on the surface of the microcarrier, while DF-1 cells in the control group have no CPE or shedding *(Fig. 4-A)*.

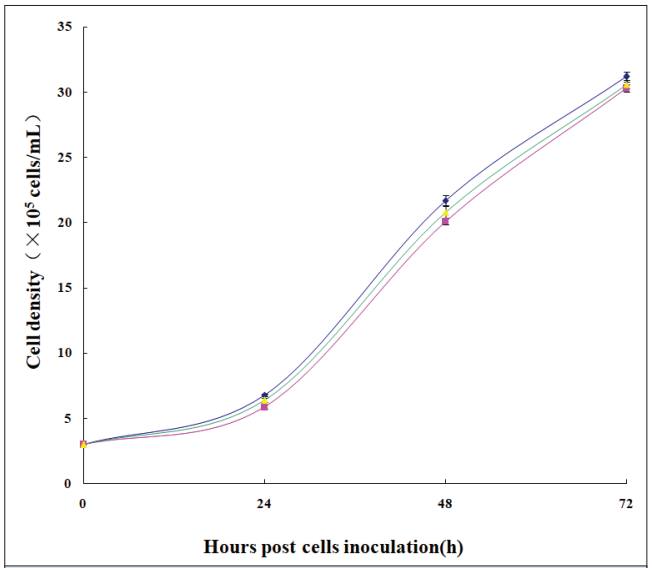

Fig 2. The DF-1 cells growth curve on Cephodex microcarrier at 37°C

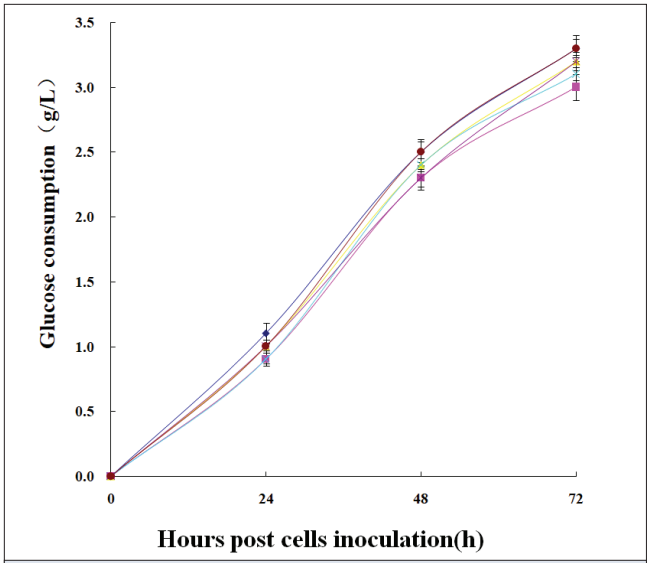

Fig 3. Glucose consumption curve of the DF-1 cells in the growth culture stage

To 72 h post CDV inoculation, the cytopathy and cell abscission further intensified, and some microspheres without cells began to appear [27]. Among the three culture temperatures, the rate of microspheres without cells at 37°C was the highest *(Fig. 4-H)*, more than 70%, followed by 35°C, nearly half of microcarriers without cells *(Fig. 4-G)*, while only a few microbeads were empty beads at 33°C *(Fig. 4-F)*. At the same time, DF-1 cells in the healthy control group were still in good condition, without CPE and obvious cell abscission, as shown *(Fig. 4-E)*. Therefore, after CDV infection, there are certain differences in the degree of cell lesions and cell abscission on the surface of microcarriers at different culture temperatures at the same time [28-30].

Cell Glucose Consumption and Virus Titer in CDV Proliferation

After the cells were infected by virus, samples were taken every 24 h to measure the viral titer. At the same time, the daily glucose consumption of cells was obtained according to measured the glucose content in the culture medium. From the results of $TCID_{50}$ determination, the viral titer showed an upward trend within 72 h at three culture temperatures, indicating that the virus maintained

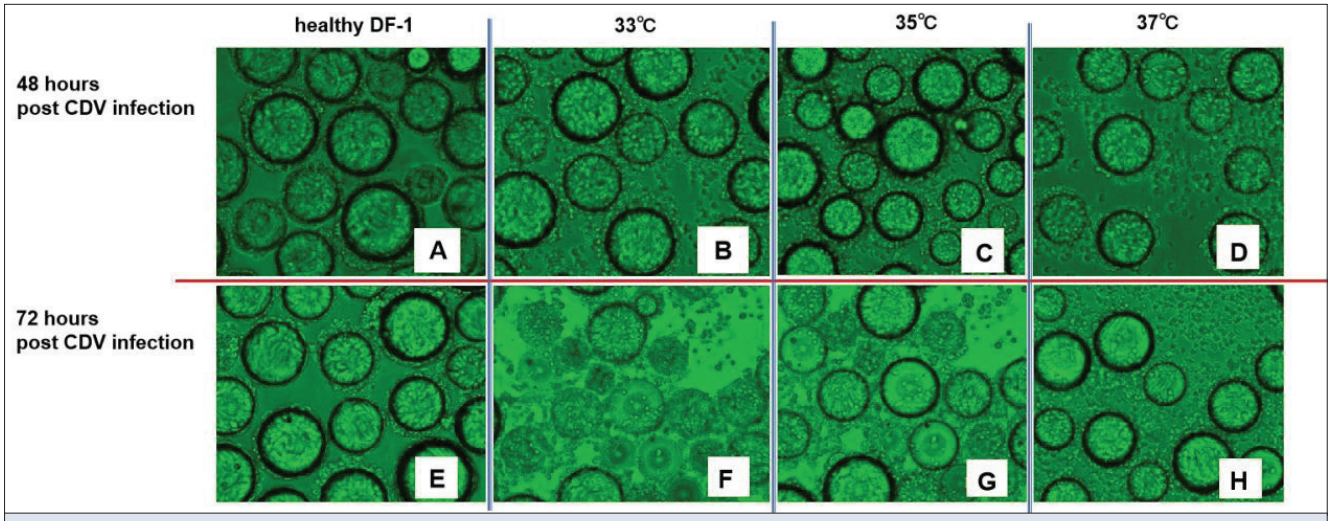

Fig 4. The CPE of DF-1 on the Cephodex after CDV infection in the different temperature. **A, B, C, D-** 48 h post CDV infection; **E, F, G, H-** 72 h post CDV infection; **A, E-** healthy DF-1 group; **B, F-** CDV infection group at 33°C; **C, G-** CDV infection group at 35°C; **D, H-** CDV infection group at 37°C

a high proliferation during this time *(Fig. 5)*. However, the virus titer at the same time was significantly different between different temperature conditions (P<0.05) *(Table 2)*. Among them, the titers of both 33°C and 37°C were lower than that of 35°C *(Fig. 6)*. Therefore, in the CDV proliferation stage, the 35°C was more conducive to CDV proliferation. The results of virus titer measurement showed that under the three temperature conditions, the viral titer increased rapidly within 48 h after virus exposure, indicating that the virus proliferated rapidly [31,32]. After 48 h, the virus titer still increased, but the rising rate slowed down slightly, indicating that the virus proliferation rate had begun to decline. From the point of view of cell glucose consumption at this stage, the rate of glucose consumption

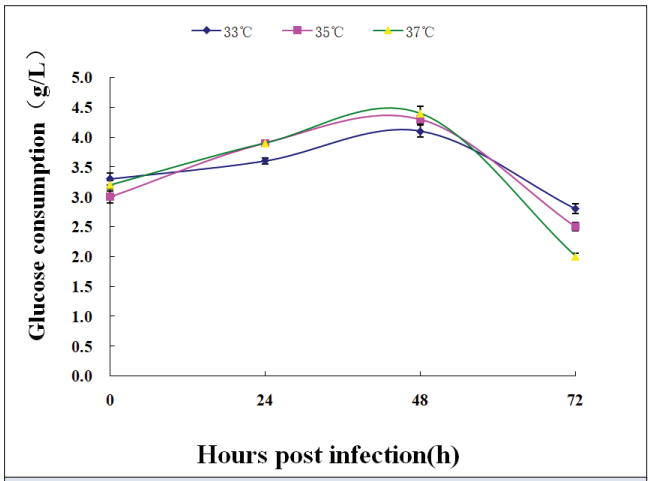

Fig 5. The glucose consumption curve of DF-1 cells infected by CDV at the different temperature

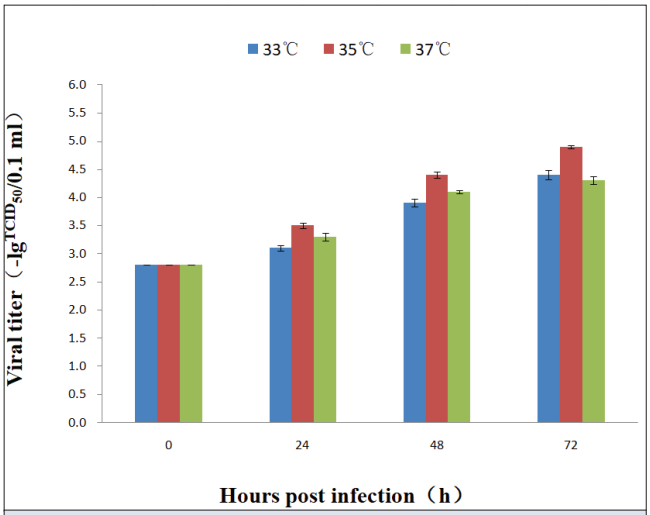

Fig 6. The viral titer in the culture at the different temperature

Table 2. *The CDV titer in different culture temperature*

Culture Temperature (°)	Culture Time				Viral Titer
	0 h	24 h	48 h	72 h	
33	2.8±0.03	3.1±0.05	3.9±0.07	4.4±0.08 ^{b}	lg^{TCID50}/0.1 mL
35	2.8±0.03	3.5±0.05	4.4±0.05	4.9±0.03 ^{a}	
37	2.8±0.03	3.3±0.07	4.1±0.03	4.3±0.07 ^{b}	

^{a,b} Values within a row with different superscripts differ significantly at P<0.05

increased continuously within 48 h after exposure, and the daily glucose consumption reached the highest level at 48 h. After 48 h, the glucose consumption of cells decreased significantly. After 72 h, the daily glucose consumption of cells in each group decreased to about half of the peak value, and the decrease was the fastest in the 37°C group *(Fig. 5)*. Therefore, the appropriate time to harvesting virus was 72 h post CDV inoculation.

Discussion

The cell compatibility of cell microcarriers is one of the key factors for the successful application of microcarrier suspension culture technology, and it is an important condition to determine whether cells can grow well in large scale and high density [33-35]. According to the results of this study, in the intermittent shaking stage, DF-1 cells adhered fast to Cephodex microcarriers, and had high adherent ratio and good morphology on Cephodex. In the continuous shaking stage, the cells had high vitality and vigorous growth on the surface of the microcarrier, forming a dense cell monolayer or multilayer structure. These data showed that Cephodex microcarrier had good compatibility with DF-1 cells and was very suitable to suspension culture DF-1 cells [36,37].

The quality and the number of cells have an important impact on the viral titer and the virus antigenicity [38,39]. For DF-1 cells growing rapidly and stably in a short time, 37°C was determined in the cell growth stage. The relationship between cell glucose consumption and cell density was investigated. The former had a significant positive correlation with the latter, and was verified in the cell counting results. After 48 h of cell culture, the growth rate of cell density and daily glucose consumption began to decline, indicating that the growth rate of cells began to slow down. The cell density reached the peak, and the daily glucose consumption almost did not increase at 72 h of cells culture which was the best time for virus inoculation. Of course, in order to determine a more accurate CDV inoculation time, we further investigated by subdividing the culture time [40]. As multiple sampling for cell counting could reduce the number of cells, it would inevitably affect the subsequent virus proliferation effect. It was little impact on virus proliferation that the supernatant was sampled to measure the glucose content because of generally no cell loss [41]. Therefore, only three shake flasks of cells were taken for cell counting and glucose consumption, which were used as healthy cell control. The other three flasks of cells for CDV inoculation were measured about the glucose consumption without cell counting. In this way, according to the daily glucose consumption of all 6 flasks of cells and the cell density in 3 flasks of control group, the cell density in 3 flasks of virus infection group could be predicted, which improved the accuracy and reliability of the experimental results. Of course, it might not be accurate to predict cell density only by cell glucose consumption which causing the experimental error, but this error was much smaller than the error led by cell loss by sampling for cell count. In order to accurately calculate the quantitative relationship between cell density and cell glucose consumption, the standard curves between them could be obtained by designed experiments in the future to reduce the error [42]. The virus titer increased rapidly within CDV inoculation 48 h, and the daily glucose consumption of cells also maintained a rapid increase. After 48 h, the glucose consumption decreased significantly, and the growth of viral titer slowed down. To 72 h the CDV proliferated, the sugar consumption decreased by nearly half, which in the 37°C group decreased especially more than 50%. Combined with microscopic observation of cytopathy, 72 h post virus inoculation could be determined as the best time for virus harvest. The virus titer at 35°C was about $10^{0.5}$ $TCID_{50}$/0.1 mL higher than that at 33°C and 37°C, which indicated 35°C was the best suitable temperature for CDV proliferation.

In conclusion, the new Cephodex microcarrier was applied to the high-density culture of DF-1 cells to CDV proliferate in this study. With a good cell compatibility, Cephodex microcarrier can be well used for large-scale culture of DF-1 cells and efficient proliferation of CDV. Moreover, CDV titer can be improved to a certain extent through the control of the key technical links, such as virus inoculation time, virus harvest time and culture temperature [43]. Since only several key technologies were discussed in the CDV suspension culture process with Cephodex microcarrier, there were more technical conditions to be optimized and improved for industrial vaccine production, which needed to be further studied in the future.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author (J. Zhuang) on reasonable request.

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Ethical Approval

The study was approved by the local Ethics Committee of Shandong Binzhou Animal Science and Veterinary Medicine Academy, Binzhou, China (Approval no: SDBZASVM-2020-002).

Competing Interests

The authors declared that there is no conflict of interest.

Author Contributions

Experimental design was conceived by Jianguo MEI, Yumao

WANG, Bing ZHANG, Shijun FU and Shijin GUO. Data were collected by Ling Mo and Lu Guo. Statistical analysis was conducted by Yan WANG and Jingjing SONG. Original draft was written by Jianguo MEI, Shijun FU and Jinqiu ZHUANG. All authors have contributed to the revision and final proof-reading of the manuscript.

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