OPTIMIZATION OF CELL CULTURE CONDITIONS FOR THE PRODUCTION OF NEWCASTLE DISEASE VIRUS

BY

MOHD AZMIR BIN ARIFIN

A thesis submitted in fulfilment of the requirement for the degree of Master of Science (Biotechnology Engineering)

Kulliyah of Engineering
International Islamic University
Malaysia

AUGUST 2011

ABSTRACT

The present work aims to prepare a model for the production of lentogenic Asplin F strain of Newcastle disease virus (NDV) using cell culture in bioreactor for live vaccine preparation. First NDV was adapted in four different cell lines namely DF-1, CEF, MSB-1 and Vero cells in T-flasks. DF-1 was found to be the most potential for NDV propagation in terms of high NDV production, rapid proliferation rate and several ethical values. Viruses produced in DF-1 cell culture were confirmed as lentogenic NDV strain by molecular diagnosis by using reverse transcriptase polymerase chain reaction (RT-PCR). In a screening experiment, high cell concentration during time of infection was found to be the most significant factor for NDV production. In another screening experiment, DF-1 was cultured in DMEM, DMEM-F12, RPMI 1640 and MEM. Culture of DF-1 cells in DMEM has the highest cell concentration of 1.240×10^6 cells/ml with specific growth rate and doubling time of 0.0180 h^{-1} and 38.4967 hours respectively. Composition of culture medium was later optimized and it was observed that culture has optimum cell concentration of 1.368×10^6 cells/ml when 2.1 g/L of $NaHCO_3$ and 7.5% serum is included in the culture medium. Experiment was continued with culture of DF-1 cells in spinner vessel. In an optimization of microcarrier concentration study, culture with 3 g/L microcarrier achieved maximum cell concentration of 0.515×10^6 cells/ml compared to culture having 1 g/L, 2 g/L, 4 g/L and 5 g/L microcarriers. Based on this result and previous optimization studies, NDV was propagated in DF-1 cell culture in spinner vessel and the maximum virus titre achieved was 128 HA unit with infectivity titre of 3.08×10^7 $TCID_{50}$/ml. In a separate experiment using 2-L stirred tank bioreactor, an optimization of process conditions study was conducted and it was analyzed that maximum cell concentration of 1.213×10^6 cells/ml will be obtained when the agitation rate is set at 71 rpm, microcarrier concentration at 2.9 g/L and pO_2 pressure at 20%. NDV was also propagated in DF-1 cell culture in stirred tank bioreactor and the maximum virus titre achieved was 4 HA unit with infectivity titre of 1.03×10^3 $TCID_{50}$/ml. Viruses produced from both types of bioreactors were later concentrated by using the high speed centrifugation method. An optimization study on process conditions of high speed centrifugation was conducted and it was revealed that optimum virus titre of 256 HA unit was achieved when the sample concentration was set at 50%, centrifugation speed was set at 12800 rpm and centrifugation time was set at 8 hours. The ability of cell culture in producing NDV in bioreactors has been shown in this study thus the model can be proposed for ND vaccine production in the future.

خلاصة البحث

هذا العمل يهدف إلى إعداد نموذج لإنتاج (Asplin F lentogenic) من سلالة فيروس مرض نيوكاسل (NDV) باستخدام تقنية في المفاعل الحيوي لإعداد اللقاح الحي. أولاً، تم تكييف NDV في أربعة سلالة مختلفة من الخلايا وهي DF - 1 ، CEF، MSB - 1 وخلايا Vero في قواريرT . DF - 1 وجدت لتكون الأكثر المحتملة لإكثار NDV من حيث الإنتاج NDV عالية ، ومعدل الانتشار السريع و عدة القيم الأخلاقية . وتأكدت الفيروسات التي نتجت من زراعة الخلايا DF - 1 كما سلالة lentogenic NDV بواسطة التشخيص الجزيئي باستخدام تفاعل البلمرة العكسية الناسخ (PCR - RT). في تجربة الفرز ، تبين أن التركيزات عالية الخلية خلال فترة العدوى من أهم عامل لإنتاج NDV. في تجربة الفرز آخرى ، زرعت DF - 1 في بيئات DMEM، F12 DMEM، RPMI 1640 و MEM. الخلايا DF – 1 المزروعة في DMEM لديه تركيز أعلى من خلية $10^6 \times 1.240$ خلية / مل مع معدل نمو محدد ووقت مضاعفة 0.0180 / ساعة و 38.4967 ساعات على التوالي. بعد ذلك، تم تحسين تركيبة البيئة المزروعة ولوحظ أن تركيز خلية البيئة الأمثل هي 1.368 x 10^6 من خلية / مل عندما يتم تضمين 2.1 غرام / لتر من NaHCO3 و 7.5 ٪ مصل الدم في خلية البيئة. في دراسة لتحسين تركيز الmicrocarrier (حاملات دقيقة)، البيئة بتركيز 3 غرام / لتر من حاملات دقيقة أعطي تركيز أعلى للخلية عند مقارنة مع البيئة بتركيز 1 غرام / لتر ، 2 غرام / لتر ، 4 غرام / لتر و 5 غرام / لتر من حاملات دقيقة بتركيز خلية حققت الحد الأقصى $10^6 \times 0.515$خلية / مل. واستنادا إلى هذه النتيجة ودراسات التحسين السابقة، NDV تكاثرت في الخلية الزارعة DF - 1 في وعاء الدوار و الحد الأقصى لعيار الفيروس الذي تحقق هي 128 وحدة HA مع عيار العدوى من 3.08×10^7 $TCID_{50}$/ml. في تجربة منفصلة باستخدام 2 لتر خزان مفاعل حيوي متحرك ، وأجريت دراسة لتحسين ظروف العملية وتم تحليلها بحصول الحد الأقصى للتركيز من خلايا $10^6 \times 1.213$ خلية / مل عند تعيين معدل الهياج في الدقيقة 71 دوره. تركيز حاملات دقيقة 2.9 غرام / لتر وPO2 الضغط بنسبة 20 ٪. تكاثرت NDV في بيئة خلية DF - 1 في خزان مفاعل حيوي متحرك خزان و أقصى عيار الفيروس الذي تحقق أقصى 4 وحدة HA مقارنة مع العدوى من عيار 1.03 x 10^3 $TCID_{50}$/ml. الفيروسات التي الناتجة من كلا النوعين من المفاعلات البيولوجية تركزت في وقت لاحق باستخدام الأسلوب تنبيذ فائق. وقد أجريت دراسة لتحسين ظروف العملية لتنبيذ فائق وتبين أن عيار الأمثل الفيروس تحقق وحدة HA 256 عندما تم تعيين تركيز العينة بنسبة 50 ٪ ، سرعة تنبيذ في 12800 دورة في الدقيقة، وكان الوقت المحدد الطرد في 8 ساعات. وقد أظهرت هذه الدراسة قدرة تقنية زراعة الخلايا في إنتاج NDV في المفاعلات الحيوية في وبالتالي يمكن اقتراح نموذج لإنتاج لقاح ND في المستقبل.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology Engineering).

_____
Maizirwan Mel
Supervisor

_____
Mohamed Ismail Abd. Karim
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology Engineering).

_____
Examiner (Internal)
Raha Ahmad Raus

_____
Examiner (External)
Fadzilah Adibah Abdul Majid

This thesis was submitted to the Department of Biotechnology Engineering and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology Engineering).

_____
Md. Zahangir Alam
Head, Department of
Biotechnology Engineering

This thesis was submitted to the Kulliyyah of Engineering and is accepted as a fulfillment of the requirement for the degree of Master of Science (Biotechnology Engineering).

_____
Amir Akramin Shafie
Dean, Kulliyyah of Engineering

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Mohd Azmir Bin Arifin

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ACKNOWLEDGEMENTS

Praises to Allah S.W.T., The Almighty God for His blessings has enabled me to accomplish the study. I would like to express the deepest appreciation to my supervisor, Assoc. Prof. Dr. Maizirwan Mel for his guidance, advises, encouragement, patience and valuable assistance that enable me to accomplish MSc. program smoothly and efficiently. I also would like to thank my co-supervisors, Prof. Mohd Ismail Abd. Karim and Assoc. Prof. Dr. Sharifah Syed Hassan for their invaluable input, time and support during my study.

I sincerely appreciate the friendship and assistance from all lab mates and lab technicians, Br. Jaafar, Sr. Siti Hajar, Sr. Yusilawati, Sr. Najiah, Sr. Alawiyah, Br. Tamrin, Br. Zulkarnain, Br. Wan Fazli, Br. Saiful, Br. Hafizul, Br. Aslan and Br. Sukiman. My special thanks go to En. Kamaruddin who had shared my burden and worked restlessly in adapting Newcastle disease virus in various cell lines. I am deeply grateful to his commitment and assistance. I wish to extend my appreciation to everyone, although not individually named here, who had contributed directly or indirectly to my project and thesis.

Last but not least, I want to take the opportunity to thank my parents, brothers and sisters for their endless love, care and encouragement. Without all of you, it would not be possible for me to complete my project and thesis. May Allah bless you all for your kindness.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
APMV-1	Avian Paramyxovirus Serotype 1
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
BCEFi	Breast-derived CEF Immortal
BHK	Baby Hamster Kidney
CAV	Chicken Anemia Virus
CEF	Chicken Embryo Fibroblast
CPE	Cytopathic Effect
CS	Calf Serum
DCU	Digital Control Unit
DEAE	Diethylaminoethyl
DF-1	Douglas Foster
DMEM	Dulbecco's Modification of Eagle's Medium
DMEM/F12	Dulbecco's Modification of Eagle's Medium Formulation-12
DNA	Deoxyribonucleic Acid
DOE	Design of Experiment
ECACC	The European Collection of Cell Cultures
EDTA	Ethylenediaminetetraacetic Acid
EID_{50}	50% Egg Infective Dose
ELISA	Enzyme-linked Immunosorbent Assay
ELL	East Lansing Line
F	Fusion Protein
FBS	Fetal Bovine Serum
FD	Full Factorial Design
FLFAM	Federation of Livestock Farmer's Associations of Malaysia
FFD	Fractional Factorial Design
G	Guanine
GE	Gene End
HA	Haemagglutination Assay
HCEFi	Heart-derived CEF Immortal
HeLa	Human Cervical Carcinoma
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane Sulfonic Acid
HIV	Human Immunodeficiency Virus
HN	Haemagglutinin/Neuraminidase Protein
HS	Horse Serum
IFN	Interferon
L	Large Protein
LD_{50}	50% Lethal Dose
M	Matrix Protein
MDBK	Madin-Darby Bovine Kidney
MDCK	Madin-Darby Canine Kidney
MDV	Marek Disease Virus
MEM	Minimum Essential Medium

MMR	Measles, Mumps, and Rubella
MOI	Multiplicity of Infection
MVP	Malaysia Vaccine Pharmaceuticals Sdn. Bhd.
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NIH	National Institute of Health
NVND	Neurotropic Velogenic Newcastle Disease
OIE	Office de Internationale Epizooties
ORF	Open Reading Frame
P	Phosphoprotein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PFU	Plaque Forming Unit
PTFE	Polytetrafluoroethylene
RBC	Red Blood Cells
RDRP	RNA-dependent RNA Polymerase
RNA	Ribonucleic Acid
RPMI	Rosewell Park Memorial Institute
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SPF	Specific Pathogen Free
TBE	Tris/Borate/EDTA
$TCID_{50}$	50% Tissue Culture Infective Dose
UPM	Universiti Putra Malaysia
UV	Ultraviolet
VP-SFM	Virus Production Serum Free Medium
VRI	Veterinary Research Institute

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Newcastle disease (ND) caused by Newcastle disease virus (NDV) is regarded as one of the most important viral diseases of poultry in the world (Spradbrow, 1987; Adene, 1990). The disease has a worldwide distribution, and is a major threat to the poultry industries due to the huge economic loss associated with it. ND which had its first outbreak in 1926, in Java, Indonesia (Kraneveld, 1926), was named after a place in England where it was rediscovered a year later (Doyle, 1927). About 27 of the 50 orders of birds have been reported to be susceptible to natural or experimental infections of NDV (Yusoff and Tan, 2001). Chickens are the most susceptible host, in which the severity of the disease may vary from mild infection with no apparent clinical signs to a severe form causing 100% mortality (Huang, et al., 2003).

NDV which is also designated as avian paramyxovirus 1 (APMV-1) is a member of the genus Avulavirus of the family Paramyxoviridae, in the order Mononegavirales (Mayo, 2002). Other important pathogens included in this family are the mumps virus, human parainfluenza virus, sendai virus, simian virus 5 and recently emerging nipah and hendra viruses. Electron microscopic examinations reveal that virus particles of members of this group have pleomorphic structure (Yusoff and Tan, 2001). They generally appear as rounded particles with diameters around 100-500 nm but often filamentous forms of about 100 nm across and variable length are seen (Alexander, 1988b).

The virus is enveloped and contains a single linear strand, non-segmented negative sense RNA molecule of 15,186 nucleotides (nt) as its genome (Krishnamurthy and Samal, 1998; de Leeuw and Peeters, 1999). The genomic RNA of the virus is consists of six genes, which encode for at least eight proteins (Peeples, 1988). The genes are arranged in tandem in the order of 3' -NP-P-M-F-HN-L- 5' which encode for nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN) and large polymerase protein (L), respectively (de Leeuw and Peeters, 1999).

Based on the clinical signs and severity of disease produced in chickens, strains of NDV are classified into three major pathotypes; lentogenic, mesogenic, and velogenic. Lentogenic strains cause mild or avirulent infections that are largely limited to the respiratory. Strains of intermediate virulence that cause respiratory disease with moderate mortality are termed mesogenic while strains that are highly virulent causing 100% mortality in chickens are termed as velogenic. Velogenic strains can be further categorized into two types: viscerotropic and neurotropic. Viscerotropic velogenic strains produce lethal hemorrhagic lesions in the digestive tract whereas neurotropic velogenic strains produce neurological and respiratory disorders (Spradbrow, 1987; Alexander, 1997).

NDV is usually transmitted through 'traditional routes' such as direct animal contact, contaminated feed, water, implements, transport contact or transmission by people (Alexander, 1988a). Airborne transmission is also considered as one of the important routes for spreading of ND. ND outbreaks in England in 1970–1971 (Hugh-Jones et al., 1973) and epidemic in Northern Ireland in 1973 (McFerran, 1989) was believed to be caused by airborne NDV. The success of NDV transmission through this route depends on many environmental factors, such as temperature, humidity, and

stocking density. Delay et al. (1948) and Hietala et al. (2005) have demonstrated that NDV can be recovered from poultry environment air samples. Although airborne NDV is a major threat to poultry farms, they are usually heat labile and readily destroyed by exposure to high temperature (>50°C) and also by ultraviolet rays in sunlight (Lomniczi, 1975).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

ND has produced huge economic losses to many economies worldwide. Before the emergence of the highly pathogenic Asian H5N1 influenza virus, the economic impact of ND has remained unsurpassed by other poultry viruses. In developed countries where the poultry industries are well established, not only outbreaks of ND are extremely costly but preventive measures taken to avoid the disease also represent continuing losses to the industry (Alexander and Senne, 2008). In 2002, an outbreak caused by a virulent strain of NDV in California, USA has caused $200 million worth of losses from the depopulation of birds (Kapczynski and King, 2005). Furthermore countries with ND free status also have to bear huge costs from repeated testing to maintain the status and for purposes of trade. While in many developing countries, recurring outbreaks has made ND a major limiting factor to the growth of commercial poultry industries and establishment of trade links (Alexander and Senne, 2008). Yusoff (2008) has reported that in Malaysia alone, the losses caused by ND amount to over RM 100 million in costs annually.

Until now, there is no treatment or method to eradicate ND. Prevention is by importing birds from disease free flocks or through vaccination that must continue throughout the life of the bird (Alexander, 1992; OIE, 2002). Traditionally, ND vaccines have been produced by growing vaccine virus strains in embryonated

chicken eggs. NDV is harvested from the allantoic fluid and processed to create a vaccine (Gallili and Nathan, 1998). This traditional method however poses some drawbacks, such as poor quality control, high labor-intensity, time consuming, needs high amount of specific pathogen-free eggs, and requires big area for the incubation of eggs. Besides the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics (Souza, et al., 2009).

In Malaysia there are only two agencies that produce vaccines for ND; the Veterinary Research Institute (VRI) and also the Malaysia Vaccine Pharmaceuticals (MVP) Sdn. Bhd. Both agencies use the cumbersome embryonated chicken eggs method which has resulted to insufficient amount and types of vaccines required to provide immunities to almost 516 million birds (broilers) produced in Malaysia in 2009 (FLFAM, 2010). To fulfill high local demand of ND vaccines, Malaysia has to import vaccines from overseas and this step poses a great risk because newer strains may be introduced to the local poultry industries.

1.3 RESEARCH HYPOTHESES

One method that has high possibility to overcome all the problems mentioned is by producing ND vaccines by using animal cell culture. Animal cell culture offers many advantages over the traditional chicken eggs method. The method is rapid, convenient, less expensive than eggs, supports easy scale up and also it allows evidence of viral proliferation to be examined microscopically. Many cell substrate systems have been reported to be able to support the growth of NDV (de Leeuw and Peeters, 1999; DiNapoli, et al., 2007; Ravindraa, et al., 2008). In addition, more mechanized cell culture methodology allows virus particles to be produced with the highest control and quality. There are also numerous types of culture medium and bioreactor systems that

are easily modified to mimic the environment of virus replication in eggs. And finally with the advent of microcarrier cell culture technology, high density cell culture is achievable and virus yield produced from the cell culture is expected to be more than the embryonated chicken eggs method or at least on par.

1.4 RESEARCH OBJECTIVES

The general objective of this study is to develop a process for producing Newcastle disease virus (Asplin F strain) for preparation of live vaccine by using cell culture as an alternative to the traditional embryonated chicken eggs method. The specific objectives based on the reasons discussed are as follows:

i. To determine the most suitable host cell line for Newcastle disease virus adaptation.
ii. To optimize Newcastle disease virus production using cell culture in bioreactor.
iii. To optimize the process conditions for Newcastle disease virus concentration using the high speed centrifugation method.

1.5 RESEARCH METHODOLOGY

Major steps involved in this study are presented in Figure 1.1. A more detailed methodology is described in chapter three of this thesis.

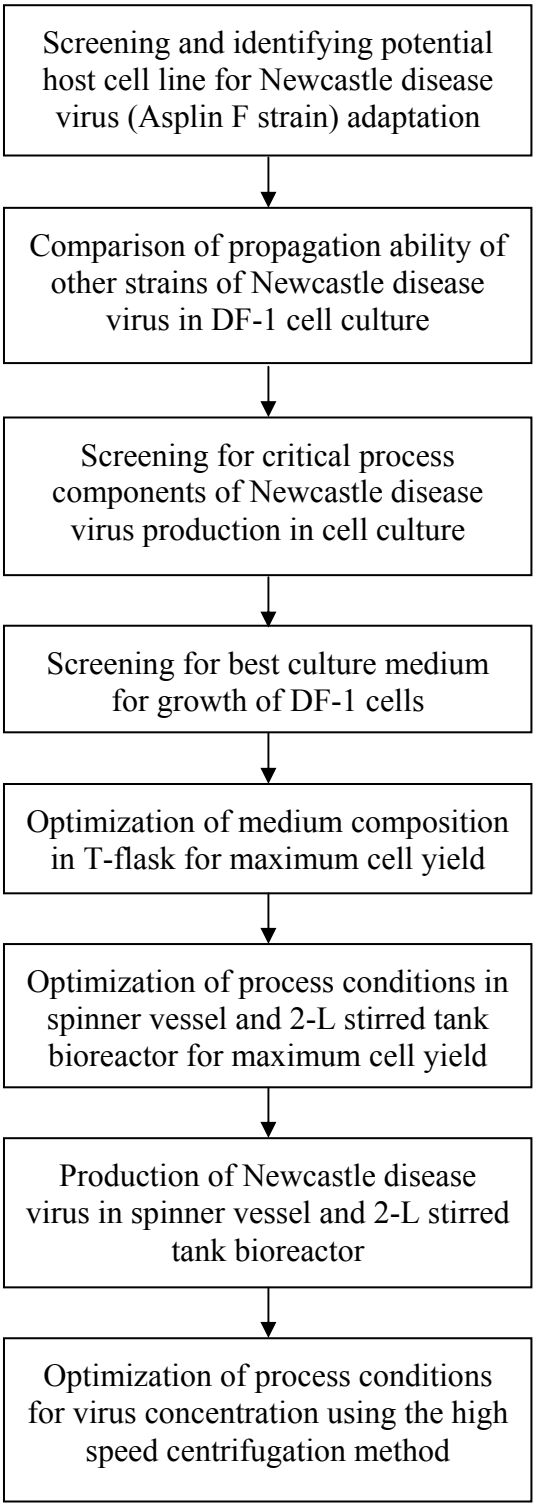

Figure 1.1: Production of Newcastle disease virus in cell culture system

1.6 SCOPE OF STUDY

The study was confined within the scope of works starting from the adaptation of Newcastle disease virus in cell culture using T-flasks to concentration of virus produced from cell culture in spinner vessel and 2-L stirred tank bioreactor by using the high speed centrifugation method. The outline of the scope is sequentially arranged as follows:

i. Adaptation of Newcastle disease virus (Asplin F strain) in several cell lines in T-flasks
ii. Medium and process optimization for maximum virus production
iii. Production of Newcastle disease virus in spinner vessel and 2-L stirred tank bioreactor
iv. Concentration of virus by using high speed centrifugation

1.7 THESIS ORGANIZATION

Chapter 1 presents the background, hypotheses, scope, and objectives of the research work. Chapter 2 reviews the available literature in the field of Newcastle disease virus production techniques. A detailed review on Newcastle disease and Newcastle disease virus has been discussed. This chapter also discusses different cell lines used in Newcastle disease virus propagation as well as the materials and equipments used in the current study. Chapter three discusses the detailed methodology of the study including materials and experimental and analytical procedures. Chapter four presents the experimental results and extensive discussion is given on the basis of results. Chapter five provides general conclusions of the research work coupled with some recommendations on how to improve the research.