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# INVESTIGATION OF THE EFFECTIVENESS OF NEWCASTLE DISEASE VIRUS PRODUCTION IN DIFFERENT BIOREACTORS

BY

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A dissertation submitted in partial fulfilment of the requirements for the degree of Master of Science (Biotechnology Engineering)

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DECEMBER 2009

# ABSTRACT

Newcastle Disease (ND) is a highly devastating poultry disease that sabotages economic growth. Currently, the traditional method of using egg in vaccine production is expensive, inconvenient to determine virus proliferation, time consuming, laborious and requires dozens of crates of egg but with low yields. The current concept of quality assurance of vaccines is less established with egg-based production. Furthermore, the relationship between freeze/thaw and sonication with respect to virus recovery is poorly documented. This study aimed at transferring the existing production process from egg to cell culture through the selection of a suitable host cell line that would sustain the fast replication of Newcastle disease virus (NDV) and the appropriate culture system that could augment maximum yield of the virus. Recovery of virus using freeze/thaw and sonication was also studied. First experiments of host cell selection for virus propagation in CEF, DF-1 and Vero cells were carried out in T-flask using both velogenic and lentogenic (AF2240 and F respectively) strains of NDV. Analysis was on fast adaptation and ethical values. As these experiments were successful, different culture media (DMEM F/12, DMEM, RPMI and MEM) selection based on cost, high viable cell concentration and virus yield was carried out using T-flask. Microcarrier selection (Cytodex 1 and 3, Hillex® and Plastic Plus®) for microcarrier culture was carried out using Spinner flask. Selection included Halal composition and high viable cell concentration. Different experiments for virus yield in different bioreactors (T flask, BelloCell, Spinner flask and Stirred tank bioreactor) were carried out using both AF2240 and F strain. Lastly, experiments generated using STATISTICA software, were carried out to examine the effect of freeze/thaw and sonication on virus recovery using a water bath sonicator. Vero cell became more adapted to AF2240 compared to other cell lines with HA titre of 2048 after three passages, but due to ethical concern, DF-1 was used. DMEM had the highest viable cell concentration with $1.25 \pm 0.032 \times 10^6$ cells/ml, but could not yield high virus titre. RPMI, however, had a viable cell concentration of $8.10 \pm 0.926 \times 10^5$ cells/ml and supported high titre of virus in addition to being more economical. Cytodex 3 had the highest viable cell concentration, $2.65 \pm 0.201 \times 10^5$ cells/ml, but had a pig gelatin surface coating. Cytodex 1, however, had a viable cell concentration of $2.03 \pm 0.217 \times 10^5$ cells/ml with no gelatin coating. Stirred tank and T-flask gave the highest HA titre of 128 using F strain of NDV. The HI titre of the propagated virus was 32 that was within the standards of OIE. One cycle of freeze/thaw, sonicating at medium amplitude for 1.5 minutes at water bath temperature of 15 $^{o}$C had the highest virus recovered. The ability to produce antigenic NDV in stirred tank could be proposed to substitute the existing egg method of NDV vaccine production in the future.

# ملخص البحث

مرض نيوكاسل (ND) مرض دواجنِ مُدَمّرِ جداً ويُخرّبُ النمو الإقتصاديَ . إضافة إلى ذلك ، الطريقة التقليدية. علاوة على ذلك ، العلاقة بين التجميدِ / ذوبان فيما يتعلق بتحسّنِ إنتاج الفيروسِ بطريقة الإتساقِ العامِّ غير موثّقُة بشكل جيد . هذه الدراسةِ إستهدفتْ تَحويل طريقةِ إنتاج اللقاح الحاليةِ التي تعتمد إستخدام البيضِ إلى طريقة تزريع الخليةِ والتي تتم من خلال إختيارِ بيئة من الخلايا تناسب وتَتحمّلُ الإستجابَة السريعَة لتكاثرفيروسِ مرضِ نيوكاسل (NDV). تمت أيضاً دراسة عمليةِ لتُحسّيَنِ إنتاجية الفيروسِ بإستعمال التجميد/ ذوبان وsonication . التجارب الأولية لإختيارِ بيئة الخلايا الملائمة لتوليدِ الفيروسِ في CEF، وDF-1 وخلايا Vero نُفّذت في الدورق (T-flask) التي تَستعمل كلا من velogenic وlentogenic (AF 2240 و F) من سلالات NDV على التوالي. التجاربُ المختلفةُ لإنتاج الفيروسِ أجريت على السلالتيتن (AF 2240 و F) في مفاعلات بيولوجية مختلفة وهي ( T flask, BelloCell, Spinner flask and Stirred tank bioreactor) . قيمت التجارب على أساس فعّالية المفاعل الحيوي في دْعمُ تكاثر الفيروسِ . أخيراً تم التحليل الإحصائي للتجارب بإستعمال برامجِ (Statistica) لفَحْص تأثيرِ التجميدِ / ذوبان وsonication على إسترجاع الفيروسِ باستخدام حمّام ماءِ (sonicator). بسبب المعايير الأخلاقية تم إختيار الخلايا (DF-1) بالرغم من أن خليةُ Vero أكثر تَكيفاً إلى AF 2240 مُقَارَنة إلى الخليةِ الأخرى تُخطّطُ مَع (HA titre) مِنْ **2048**. المفاعلين (Stirred tank) و الدورق (T-flask) أعطيا الإنتاجية الأعلى من (HA titre) مِنْ **128** بإستعمال الســـلالة F من NDV . ال (HI titre) للفيروسِ المُكَاثَرِ كَانَ **32** وذلك ضمن معاييرِ OIE. القدرة على إنْتاج لقاح لفيروس NDV في (Stirred tank) يُمْكِنُ أَنْ تُقتَرحَ كبديل مناسب لطريقةِ البيضِ المستخدمة حاليًا لإنتاج هذا اللِقاح .

# 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 dissertation for the degree of Master of Science (Biotechnology Engineering).

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# DECLARATION

I hereby declare that this dissertation 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.

Ja’afar Nuhu Ja’afar

Signature _____ Date _____

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# DECLARATION OF COPYRIGHT AND AFFIRMATION OF FAIR USE OF UNPUBLISHED RESEARCH



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Signature Date

# ACKNOWLEDGEMENTS

Alhamdulillah, all praise and glory be to Allah for making things easier for me in my life.

My parents, without whose encouragement, I would not have been here. I thank them for their unflinching prayers.

I acknowledge with thanks the invaluable assistance rendered by Prof. Ribadu and his family.

My profound gratitude goes to my able supervisor, Assoc. Prof. Dr. Maizirwan. He has been very understanding. Jazakallahu khairan. To my co-supervisors, Prof. Dr. Mohamed Ismail Abdulkarim and Prof. Datin Paduka Dr. Aini Ideris, I say may Allah continue to increase you in knowledge and health.

So many people (both staff and students) are in the queue to be thanked and acknowledged. Trying to thank them individually would mean to risk leaving someone by mistake. I therefore say thank you to all of you. However, my sincere thanks go to my lab mates, Azmir *et al.*

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| | |
|---|---|
| APMV-1 | Avian Paramyxovirus Serotype 1 |
| ATCC | American Type Culture Collection |
| BVDV | Bovine Viral Diarrhoea Virus |
| CAF | Chorioallantoic fluid |
| CEF | Chicken Embryo Fibroblast |
| CHO | Chinese Hamster Ovary |
| CPAE | Cow Pulmonary Artery Endothelium |
| CPE | Cytopathic Effect |
| CVD | Crystal Violet Dye |
| DEAE | Diethylaminoethyl |
| DF-1 | Doug Foster |
| DMEM | Dulbecco's Modification of Eagle's Medium |
| DMEM/F12 | Dulbecco's Modification of Eagle's Medium Formulation-12 |
| dsRNA | Double Stranded RNA |
| DVS | Department of Veterinary Service |
| $EID_{50}$ | Egg Infective Dose |
| F | Fusion Protein |
| FBS | Foetal Bovine Serum |
| FCS | Foetal Calf Serum |
| HA | Haemagglutination |
| HeLa | Human Cervical Carcinoma |
| HEPES | N-2-Hydroxyethylpiperazine-N'-2-ethane Sulfonic acid |
| Hl | Haemagglutination Inhibition |
| HN | Haemagglutinin/Neuraminidase Protein |
| IBD | Infectious Bursal Disease |
| ICPI | Intracerebral Pathogenicity Index |
| $ID_{50}$ | Infective Dose |
| IFN | Interferon |
| IRF9 | Interferon Regulatory Factor 9 |
| ISG | Interferon Stimulated Gene |
| ISRE | Interferon Stimulated Response Elements |
| JAK1 | Janus Kinase 1 |
| L | RNA directed RNA Polymerase Protein |
| $LD_{50}$ | Lethal Dose |
| M | Matrix Protein |
| MEM | Minimum Essential Medium |
| MRC-5 | Human Embryonic Lung |
| N | Nucleoprotein |
| NB41A3 | Mouse Neuroblastoma |
| ND | Newcastle Disease |
| NDV | Newcastle Disease Virus |
| OIE | Office de Internationale Epizooties |
| P | Phosphoprotein |
| PBS | Phosphate Buffered Saline |

| | |
|---|---|
| PC | Proprotein Convertase |
| RNA | Ribonucleic acid |
| RPMI | Rosewell Park Memorial Institute |
| SPF | Specific Pathogen Free |
| STAT1 & 2 | Signal Transducers and Activators of Transcription 1 & 2 |
| $TCID_{50}$ | 50% Tissue Culture Infective Dose |
| TYK2 | Tyrosine Kinase 2 |
| USDA/APHIS | United States Department of Agriculture/Animal and Plant Health Inspection Service. |

# CHAPTER ONE

# INTRODUCTION

## 1.1 BACKGROUND

Newcastle disease (ND) is reported as the most important viral disease of poultry in the world including developing countries (Adene, 1990; Spradbrow, 1997). It has a devastating effect on commercial as well as village poultry industries (Adene, 1997). The causative agent, Newcastle disease virus (NDV), is synonymous with Avian Paramyxovirus type 1 (Russell & Alexander, 1983; Alexander, 2003) but with distinct antigenic subtypes (Alexander, Manvell, Lowings, Frost, Collins, Russell & Smith, 1997; Roy, Venugopalan & Koteeswaran, 2000). It has been classified in the order Mononegavirales, family Paramyxoviridae, subfamily Paramyxovirinae, and genus Avulavirus (Mayo, 2002; Kwon, Cho, Ahn, Seo, Choi & Kim, 2003; Liu, Wan, Ni, Wu & Liu, 2003; Lee, Sung, Choi, Kim & Song, 2004). This family also includes other important pathogens such as Mumps virus, Human Parainfluenza virus, Sendai virus, Simian virus 5 and recently emerging Nipah and Hendra viruses. Paramyxoviridae are generally spherical and ranged between 150 to 350 nm in diameter, but could be pleomorphic in shape, and filamentous forms could be observed (Condit, 2007; Lamb & Parks, 2007).

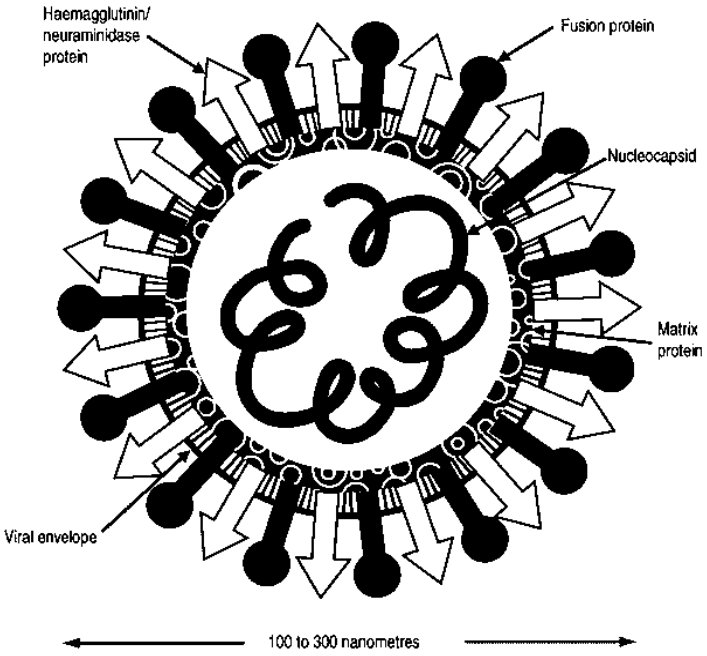


Figure 1.1 Schematic Diagram of Newcastle Disease Virus (Grimes, 2002)

Newcastle disease virus infects 8000 species of pet and free-living birds in addition to domestic avian species (chicken, turkey, goose, duck, and pigeon (Kaleta & Baldauf, 1988)). Among domestic birds, chickens are the most susceptible, whereas ducks and geese are the least susceptible (Wakamatsu, King, Kapczynski, Seal & Brown, 2006). A wide range of avian and non-avian species act as reservoirs of NDV and transmit the disease to susceptible birds (Roy, Venugopalan, Selvarangam & Ramaswamy, 1998). Contaminated faeces is a source of infection for susceptible birds, since the virus can survive for more than 8 weeks in hot dry tropical areas at temperatures of 40 $^{o}$C (Warner, 1989) and for 3 months at 20 – 30 $^{o}$C (Lancaster,1966). There is currently no treatment for the disease (*Office Internationale des Epizooties* [OIE], 2000). Prevention is to import birds from disease free flocks only (Alexander, 1992) or through vaccination that must continue throughout the life of the bird (OIE, 2000).

In chickens, ND often causes high or total mortality in the flock. Chickens may die without showing any clinical signs of infection. Once introduced into a poultry flock, the virus spreads from farm-to-farm by the movement of inapparently infected poultry species, on contaminated objects such as boots, sacks, egg trays, crates or by flies (Bram, Wilson & Sardesai, 1974).

The first recorded case of ND was from an outbreak near Newcastle-upon-Tyne, England, in 1927 were it got its name although reported earlier in Java, Indonesia in 1926 (Alexander, 1992). Infected birds may show symptoms of nervous, respiratory or digestive system disorder (United States Department of Agric/Animal and Plant Health Inspection Service [USDA/APHIS], 2003). A country is considered free of ND when the disease is absent for at least 3 years (Terrestrial Animal Health Code, 2007).

Outbreaks of ND are unpredictable; therefore discourage local farmers from paying proper attention to the husbandry and welfare of their chickens. In much of Asia, India specifically, ND is known as Ranikhet disease (Alders & Spradbrow, 2001).

Department of Veterinary Services, Malaysia, recorded a value of 185 million as the total number of chickens in the country (Department of Veterinary Services [DVS], 2006). Recent records from OIE (OIE, 2007) showed the statistics of birds in Malaysia as 220 million. However, with a projected 15% increase per annum, estimated population in 2009 will be over 290 million. For optimum protection against ND, it is recommended that broilers have two doses while layers and breeders have a minimum of three doses of ND vaccine. Therefore, more than 600 million doses of the vaccine are needed.

Currently, local pharmaceutical companies produce commercial ND vaccine using traditional and cumbersome methods. To overcome the shortage, the vaccine has to be imported from Taiwan and Thailand based on Department of Veterinary Service's statistics, thus introducing newer strains to the existing ones. This makes control of the disease more difficult.

Vaccine strains of NDV (Spradbrow, 1987) include Avirulent: causes no disease; Lentogenic: low virulence, low mortalities, and loss of egg production; Mesogenic: moderate virulence, mortalities up to 50%, loss of egg production; Velogenic: high virulence, severe disease with high mortalities. Incubation period varies with the strain of the virus, and is generally 4-6 days (range 2-15 days) (Alexander, 2004). Most of the vaccines are produced conventionally by propagating the virus in egg (Grimes, 2002).

Viruses are obligate intracellular parasites that require living cells in order to replicate. Cultured cells, eggs and laboratory animals are used for virus isolation. Although embryonated eggs and laboratory animals are very useful for the isolation of certain viruses, cell cultures are the sole system for virus isolation in most laboratories nowadays.

Eggs are relatively cheap and readily obtainable as compared to animals, which were the substrates for early virus studies. The egg has a variety of cells and membranes susceptible to infection by different viruses and can be kept in controlled stable environment. While the egg supports the replication of a variety of virus strains, methods for infecting the eggs and maintaining virus growth are time consuming, cumbersome and lack proper quality control measures.

Specific pathogen free (SPF) eggs to be used for human and animal vaccine production must be free of the following pathogens: Avian adenovirus group1; Egg

drop syndrome virus; Avian encephalomyelitis virus; Infectious bursal disease virus; Avian infectious bronchitis virus; Influenza A virus; Avian infectious laryngotracheitis virus; Marek‘s disease virus; Avian leucosis virus; Newcastle disease virus; Avian nephritis virus; Turkey rhinotracheitis virus; Avian orthoreovirus; Avian reticuloendotheliosis virus; Chicken anaemia virus; *Mycoplasma gallisepticum; Mycoplasma synoviae* and *Salmonella pullorum* (Jungbäck & Motitschke, 2008).

Cell cultures on the other hand, can be kept in a highly controlled environment as compared to eggs. They are more convenient and less expensive than eggs and animals, and are convenient to examine microscopically for evidence of viral proliferation. For many years, cell culture has served as the “gold standard” for virus detection to which all other methods have been compared (Hsiung, 1984); also provided a desirable environment for the detection and identification of many human viral pathogens (Leland & Ginocchio, 2007). However, there are still virus strains that appear to grow better in embryonated egg membranes than in cultured cells such as Hepatitis B virus (HBV), Hepatitis C virus (HCV), Hepatitis D virus (HDV), Human papilloma virus (HPV) (Cann & Irving, 1999). Newcastle disease viruses on the other hand, are usually cultivated using embryonated chicken eggs. Some strains kill the embryos; others do not. The virus will also grow in cell cultures of avian origin, and in some mammalian cells. Not all strains of NDV are cytopathic and detection of these strains in cultured cells can be difficult (Spradbrow, 1987).

The propagation of NDV has been demonstrated in tissue culture systems using chicken embryo fibroblast (CEF) cell line (Ahamed, Hossain, Billah, Islam, Ahasan, & Islam, 2004). However, these data are limited to small-scale production and do not provide a means of achieving large-scale production of NDV particles for vaccine purposes.

## 1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

Newcastle Disease (ND) is a highly devastating poultry disease that sabotages the economic growth in the agricultural sector of the country. In Malaysia, ND vaccine production is by using the egg method (Malaysian Vaccine Pharmaceutical Sdn Bhd, 2009), which is insufficient therefore the need to import. The importation poses the risk of introducing newer strains of the virus. This makes eradication of the disease difficult because of differences in virus strains used for vaccine production.

In addition, the traditional method of using egg is expensive, less convenient to determine proliferation of virus, time consuming, laborious and requires the use of dozens of crates of egg but with relatively low yields. The dose of ND vaccine required to protect the teeming population of birds in the country is over 600 million. Egg-based vaccine production produces, on the average, a dose from one or two eggs and the entire production last several months. Logically, eggs required to produce the adequate doses of the vaccine will be more than a billion eggs. The birds to produce such eggs are facing ND as a challenge to contend with. Obvious disadvantage of egg based vaccine production is the extensive planning (procurement of many million eggs, long timeline) which limits the flexibility in case of exponentially increasing demand. A Newcastle disease pandemic could probably not be contained and defeated on egg-based production, because the production takes too long and eggs do not grow on demand.

The current concept of the quality assurance of vaccines is based on the overall consistency of production, involving several in-process controls, rather than simply on a single lot release assay. The adherence to good manufacturing practice is therefore of critical importance in establishing a confidence in production process. This protocol, however, is not well established when egg based production is employed.

Identification of the above problems led to the search of alternatives to improve NDV propagation in other culture systems for vaccine production with a view to substituting the existing method of virus propagation in eggs, thereby ameliorating importation and quality control issues.

## 1.3 RESEARCH HYPOTHESIS

Viruses are obligate parasites that require a living host for their survival and replication. To propagate large amount of virus for vaccine production, continuous and consistent substrate availability is very critical. Eggs that serve as substrates for virus propagation are limited and scarce. Animal cells are other substrates that support viral replication and propagation. High number of cells will translate to high virus yield. At the laboratory scale, a 25 $cm^2$ flask can produce a total cell number of $1x10^6$ cells in two days (Butler, 2004). This has the ability of producing 5-fold yield of virus when compared to an egg. High-density cultures such as microcarrier culture for example can be up-scaled to produce cells at a range of $1x10^6$ to $1x10^7$ cells/ml (Trabelsi, Rourou, Loukil, Majoul & Kallel, 2005). Microcarrier culture can therefore be tested for NDV propagation with a view to substituting the traditional production method.

## 1.4 RESEARCH OBJECTIVES

The objectives of this research are:

i. To determine the haemagglutination (HA) titres and adaptability of different host systems to NDV.

ii. To investigate the effectiveness of producing NDV using different types of culture systems (T-flask, BelloCell, Spinner flask and Stirred tank reactor)

iii. To improve NDV extraction using sonication process

iv. To evaluate the antigenicity of the propagated virus by determining its haemagglutination inhibition (HI) titre and comparing it with *Office Internationale des Epizooties's* (OIE) HI titre for vaccine production.

## 1.5 RESEARCH METHODOLOGY

The research was a laboratory-based experimental work. It started with a literature survey. Normal host cells were maintained using T-flask culture. Host cell selection for onward virus propagation was carried out. Culture media and microcarrier selection from a range of four different media and microcarriers respectively were performed. Culture system performance was carried out by determining the growth profile of selected host cell. Virus yield from the different culture systems were determined and antigenicity of the virus produced was evaluated. Experiments for virus recovery using freeze/thaw and sonication were designed and evaluated using STATISTICA software. Writing of the research findings was the last aspect of the research. Detailed methodology is described in chapter three of this research work.

## 1.6 SCOPE OF RESEARCH

This research was to propagate NDV using a host cell line in different types of bioreactor. Antigenicity of propagated virus for vaccine development was determined using standard HA and HI tests recommended by the OIE. Sonication was the method of virus recovery from cultured cells.