IBDV 3529/92 SEGMENT A ORF 2 GENE ANALYSIS AND EXPRESSION STUDIES IN *Pichia pastoris*

BY

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ABSTRACT

Infectious Bursal Disease Virus (IBDV) outbreak had been reported to infect broiler chicken in Malaysia and causes high mortality. It had been reported that the virulence and pathogenicity of the virus is related to its coat protein, more specific the VP2 hypervariable regions. The study aimed to determine the mutation occurred in the amino acid sequence which responsible for the outbreak of the disease and increased in the virus virulence. To study this, the nucleotides and protein of the local IBDV isolate was sequenced and analyzed by comparing it with other IBDV that had been reported previously. To obtain the virus sequence, local IBDV isolate strain 3529/92 was propagated in Specific Pathogen Free eggs and RNA was extracted and amplified by RT-PCR before being inserted into pCR2.1 TOPO TA vector for cloning and sequencing. The full length of the gene segment of the coat protein was constructed by concomitantly joining the fragments using the IBDV native restrictions sites in *E. coli* expression vector, pTrchis2a. The sequence analysis revealed that the local IBDV 3529/92 consists of 3039 nucleotides which encodes for 1012 amino acids. BLAST analysis of the nucleotide sequence showed that the local strain shared the greatest similarities with Dutch D6948 very virulent (vv) IBDV subtypes. Analysis of the VP2 variable regions of 3529/92 showed most of amino acid substitutions occurred at VP2 variable region are very similar to the changes in VP2 variable regions of the vvIBDV subtypes. Phylogenetic analysis showed that 3529/92 isolates belong to the vvIBDV subtype. Recombinant plasmid was constructed and inserted into the yeast expression vector, pPICZ A prior to transformation into *Pichia pastoris X33* by electroporation. After the induction of *P. pastoris* transformant with 0.5% methanol, the production of IBDV polyprotein was observed using Western blot. In *P. pastoris*, co- or post-translational processing of large polyprotein had occurred generating a stable C-terminal product (VP3).

ملخص البحث

ان اندلا ع وانتشار مرض داء الغمبورو او الجرابي المعدي ادى اصابة و هلاك الدجاج بنسب عالية في ماليزيا.ان العديد من التقارير تشير الى ان ضراوة وامراضيه فايروس الغمبورو يعود الى احتواء الفايروس على بروتين مغلف وبشكل ادق فان السبب يعود لوجود مناطق متغايرة مفرطة)VP2(. ان هذه الدراسة تهدف الى تحديد الطفره الوراثيه في تسلسل الاحماض الامينية المسؤولة عن زيادة ضراوة الفايروس وانتشاره بشكل كبير. ولتحديد الطفره فأن النيكوليدات والبروتين العائد للفايروس المعزول محاليا قد سلسلت وحللت بواسطة مقرانته مع عزلات اخرى للفايروس والتي تم تحليلها مسبقا.من اجل للحصول على تسلسل الاحماض الامينية، تمت مكاثره عزلة محليه للفايروس لسلالة)3529/92(في بيوض محدده خالية الامراضية)SPF(وكذلك تم استخلاص وتضخيم ال RNA بواسطة RT–PCR قبل ادخاله في الناقل pCR2.1 TOPO TA من اجل استنساخه. وقد تم بعد ذلك عمليه بناء قطعة من جين كامل للبروتين المغلف بواسة ربط قطع الجين عن طريق مواقع القيود الاصلية للفايروس في الناقل pTrchis2a *E. coli*.ان تحليل تسلسل الاحماض الامينية كشفت ان العزلة المحلية تتكون من 0303 نيوكليوتيد والمسؤولة عن تشفير 0301 حامض اميني. ان تحليل البلاست لتسلسل الاحماض الامينية اظهرت ان هناك تشابه كبير بين العزلة المحليه وعزلة D6948 الهولندية الضارية جدا . وفي نفس السياق فأن تحليل المناطق المتغايره)VP2(العائدة للسلالة المحلية اظهرت ان التبديل الحاصل في الاحماض الامينية للسلالة المحلية)3529/92(مشابه لحد كبير لتلك الحاصلة في مناطق التغير لسلاسة الهولندية الضارية)vvIBDV(. اضافة الى ذلك فان تحليل النشوء والتطور كشفت ان السلاسة المحلية المعزولة تعود الى السلاله الهولنديه الضارية)vvIBDV(. لاحقا تمت عملية بناء عاثي مؤتلف وادخاله في الناقل الخميري pPICZ A قبل تحويله الى *Pichia pastoris X33* بواسطة electroporationوبعد استحثاث*P. pastoris* transformant مع 3.0 % من محلول الميثانول قد لوحظ البولي بروتين بواسطة Western blot وان عملية (بعد او قبل ترجمة البولي البروتينات الكبيرة) ل *P. pastori* قد احدثت توليد (VP3)مستقر.

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 Health Sciences.

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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.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degree Celsius
A_{260}	absorbance at 260nm
A_{280}	absorbance at 280nm
APS	ammonium persulphate
BLAST	Basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
Da	dalton
DEPC	Diethylpyrocarbonate
dH2O	Distilled water
DNA	deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
et al.,	and others
EtOH	Ethanol
g	Gram
g/L	gram per liter
h	Hour
HCl	Hydrochloric acid
IBD	Infectious Bursal Disease
IBDV	Infectious Bursal Disease Virus
kb	kilobase
kDa	kilo dalton
LB	Luria Bertani
mAbs	Monoclonal antibodies
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
ms	millisecond
MW	molecular weight
NCBI	National Center for Biotechnology Information
OD	optical density
OD_{600}	optical density at 600nm
ORF	Open Reading Frames
pAbs	polyclonal antibodies
PCR	Polymerase chain reaction
pmol	picomole
PMSF	phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
rpm	rotation per minute
RT	room temperature

RT-PCR	Reverse transcriptase polymerase chain reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ta	Annealing temperature
TAE	Tris, acetic acid, EDTA
Taq	*Thermus aquaticus*
TEMED	N,N,N, N-Tetramethylenediamide
Tm	Melting temperature
U	unit
UV	ultraviolet
V	Voltage
v/v	Volume per volume
vol	volume
w/v	weight per volume
µg	Microgram
µl	Microlitre
µM	Micromolar

CHAPTER 1

INTRODUCTION

Infectious Bursal Disease (IBD) or 'Gumboro' disease is the disease that affects young chicken (*Gallus gallus*) of the age between 3 to 6 weeks after hatching; the ages where the bursa of Fabricius is most susceptible to the disease agent, infectious bursal disease virus (IBDV). The virus targeted the lymphoid cells in the bursa of Fabricius, the main organ that plays vital role in the chicken immune defense system. Once infected the virus multiplying and infecting the actively dividing and differentiating B-cell lineage result in serious immunosuppression. Thus, most of the mortality was either because of the acute form of the infection itself, or when not fatal the death was mainly due to severe immunosuppression which increases the susceptibility to other etiological agents and an ineffective response toward vaccines. Since the emergence of the virus and before 1985, the virulence of IBDV is relatively low and cause only about 1-2% of specific mortality and the disease were satisfactorily controlled by vaccination.

The vaccination for controlling the outbreak of the disease was primarily achieved by maternally-derived antibodies induced by live and inactivated vaccines given to breeder hens or by vaccinating chicks with a live-attenuated strain of IBDV. The emergence of the new class of pathotypic variable with high mortality designated as very virulent IBDV (vvIBDV) had been reported starting in Europe (after the year 1988) and then it was subsequently widespread to Asian country including Malaysia starting from the year 1992. The main differences between the classical IBDV and vvIBDV pathotype is the ability of the vvIBDV to manifest its infection in the

presence of high maternally derived antibody which previously protective against virus infections and have a higher mortality rate. The emergence of the new pathotype of IBDV able to bypass the traditionally employed vaccination programmed show the continuous need of research and effective vaccine development and its application strategy to be explored.

IBDV the causative agent of the disease belongs to family birnaviridae, prototype virus of the genus avibirnavirus. Birnaviridae in general is double strand RNA (dsRNA) virus and all members share same virion properties. Morphologically, the virions are about 60 nm in diameter, single shelled non enveloped icosahedra and capsid structure based on T=13 lattice. In terms of nucleic acids, the virions consist of two segments of dsRNA namely segment A and segment B. Segment B contain one 94 kDa product (ORF 3), mainly codes for the viral RNA-dependent RNA polymerase. Segment A contains two ORF; ORF2 and ORF 1. ORF 2 encoding a large polyprotein of about 105 kDa and according to genome organization is in the order of 5' VP2-VP4-VP3 3'. Through a series of proteolytic cleavage the precursor polyprotein gives rise to two structural proteins VP2 and VP3, and an autoproteases VP4. ORF 1 encoding for a small non-structural peptides VP5. The major capsid protein VP2 is the main host-protective antigen against IBDV and become the major interest in producing subunit vaccines to protect animal from IBDV infections as it contain the neutralization site. The VP3 protein, aside from being the structural protein, it is reported to be an important factor for the correct assembly of the virus protein.

In this study, the IBDV segment A ORF 2 from a local field isolated was cloned and characterized. The characterizations of the IBDV from the field isolate are important in giving a better understanding about the molecular evolution and the

origin of the virus. The virus that was used is from IBDV outbreaks in Malaysia, specifically in a farm in Tronoh, Perak occurred in 1992. The virus is classified as vvIBDV as the mortality level reaches almost 100% in the infected chicken apart from other factor such as their restriction pattern when treated with certain restriction enzymes. As one of the earliest reported vvIBDV case in Malaysia it presents us an opportunity in studying and analyzing the segment A ORF 2 of the virus and compared it with other reported IBDV strains worldwide to find the changes at the molecular level. By doing this, a better understanding on how mutation contributes in increasing the virus virulence can be studied and served as an important foundation in which better vaccine can be engineered. Apart from that, during the same year, the emergence of the vvIBDV outbreaks can be observed in other Asian country such as Japan. So by studying the sequences, the correlation of the virus with other strains can be studied whether they are locally evolved or shared the same origin. Expression of the segment A ORF 2 in *P. pastoris* was also investigated for development of virus-like particles for use as a subunit vaccine.

The expression of Segment A as a whole or only VP2 had been reported in various expression systems ranging from animal cells to bacterial cells, but there is a lack of reporting on the expressions of the whole ORF 2 in *P. pastoris*. *P. pastoris* is viewed as one of the excellent expression system due to the ease of use and the ability to do post-translational process comparable to higher eukaryote which makes most of protein expressed functional. *P. pastoris* is also superior compared to other yeast expression systems, for example *Saccharomyces cerevisae* as they do not tend to overglycosylated the protein they expressed. Thus, the study of the expression of Segment A gene (ORF 2) is hoping to give a foundation in assessing the feasibility of developing new vaccine candidates against IBDV.

The objectives of this project are to:

1. To isolate the IBDV 3529/92 Segment A (ORF2) gene from the viral genome and inserted it into a cloning vector.

2. To analyze and compare IBDV 3529/92 Segment A (ORF 2) nucleotide and protein sequence with other virus isolates.

3. To investigate the expression of Segment A gene of infectious bursal disease virus (IBDV) in *Pichia pastoris*.

CHAPTER 2

LITERATURE REVIEW

2.1 INFECTIOUS BURSAL DISEASE (IBD)

Infectious Bursal Disease (IBD) or 'Gumboro disease' is one of the most economically important diseases that affect the chicken rearing industries worldwide. It is an acute, immunosuppressive and very contagious disease. The etiological agent of the disease is known as infectious bursal disease virus (IBDV). The virus (Gibbs *et al.*, 1995) infected and subsequently destroyed the bursa of Fabricius, the unique organ and the only place in avian species where the somatic gene conversions for the antibody repertoire differentiation take place (Davison *et al.*, 2008). The destruction of the bursa of Fabricius during the birds' early developments, if not fatal, makes the produced antibodies useless as they are incapable of eliciting specific antibody against new pathogen attacks. So, the mortality in affected chickens is mostly caused either through the fatal infections by the IBDV itself or in mild infections the death is as a result of the inability of the host to respond effectively towards subsequent attacks by other disease etiological agents.

The infected chicken also had reduced responses towards vaccination thus making them more susceptible towards upcoming pathogen attack. The fatality of the virus infections is depending on various factors and most prominent of them are, a) the dose and virulence of the infecting strain; b) the breed, age and the susceptibility of the chicken flocks; c) intercurrent primary and secondary pathogens; d) the farm management and environmental factors; and the presence or absence of passive immunity. There is no evidence exist regarding the transmissions of IBDV to host

other than avian species, including human. Thus the disease had no direct impact towards public health.

2.1.1 History, Field Evolutions and Epidemiology

The acute morbidity and mortality caused by a syndrome known as “avian nephrosis” were first recognized in a broiler farm near Gumboro, in the Delaware area in the USA by Cosgrove (Lasher & Davis, 1997). The outbreaks of the disease were also known as “Gumboro disease” in reference to the geographic area in which it is first discovered. The disease was first known as avian nephrosis or ‘nephritis-nephrosis syndrome of chickens’ but the name changes to Infectious Bursal Disease as it becomes evident that the main features of the disease is the enlarge bursa of Fabricius couple with the edematous swelling and hemorrhagic lesion.. The misconception of first naming the syndrome was primarily due to the available diagnostic technique during that time failed to differentiate between two concurrent infections. In the USA, the disease was rapidly spread and in 1964, all of the 13 southeastern poultry producing states had reported IBD cases and by 1965 the disease had been detected in all US broiler and egg production area (Lasher & Davis, 1997). The disease was then reported to be detected in European country starting from the year 1962 to 1971 (Faragher, 1972; Kibenge *et al.*, 1988). The disease was then widespread in the Middle East, Africa, the Far East and Australia (Pattison *et al.*, 1989; Nunoya et *al.*, 1992).

2.1.2 Variations of IBD: Antigenic and Virulence

The vaccinations programmed employed had satisfactory controlled of the outbreaks of the diseases up until 1985. But since then, the emergence of antigenic and

pathogenic variant of new strains of IBDV had been described all around the world. Starting in the US, the new strains which were believed caused by a major antigenic shift in serotype 1 was described. These viruses isolate designated as antigenic "variants" of serotype 1 IBDV. They can evade and manifest their infections, although the broiler chicken possessed a high level of maternal antibodies which previously protective against the classic strains of IBDV and were highly immunosuppressive. The "variant" types IBDV were also different than the "classic" types as the isolates caused rapid atrophy and minimal inflammation of the bursa. These new strains were reported to be responsible for up to 5% of specific mortality in chicken (Rosenberger & Cloud, 1986).

In other countries, starting with Europe in 1987, followed by Japan (1992) and other Asian country, the new pathotypic variants of IBDV designated as very virulent IBDV (vvIBDV) were described. Although, they are antigenically similar to the classic strain of IBDV (Brown, Green, & Skinner, 1994) but somehow they were able to cause the disease in the presence of high level protective maternal antibody like the "variant" strain IBDV. They were characterized by high mortality rates of up to 100% in SPF chicken and peracute onset of severe clinical disease (Ignjatovic, 2004).

2.2 INFECTIOUS BURSAL DISEASE VIRUS (IBDV)

IBDV belongs to family birnaviridae, prototype virus of the genus Avibirnavirus. The family, birnaviridae was established in 1986 in order to describe and classify a group of animal viruses that carry a bisegmented dsRNA genome as their distinguishing characteristic. Other type species that belong to this family are Infectious Pancreatic Necrovirus Virus (IPNV), from genus Aquabirnavirus, the type species which only infected fish, mollusk and crustaceans. Other type species are Drosophila X Virus,

genus Entomobirnavirus in which the member of the genus which infect insect only (Table 2.1).

Table 2.1
Genus in family birnaviridae**.**

Genus:	Type species
Avibirnavirus	Infectious Bursal Disease Virus (IBDV)
Aquabirnavirus	Infectious pancreatic necrosis virus (IPNV)
Entomobirnavirus	Drosophila X virus (DXV)

IBDV can be classified according to their serotype and virulence. The serotype was distinguished by cross-neutralization and cross-protection test. The serotype 1 is pathogenic while serotype 2 is nonpathogenic (Cummings *et al.*, 1986). The pathogenic serotype 1 can be further subdivided into classical virulent, antigenic variant or very virulent isolates.

2.3 ECONOMIC IMPACT

The economic loss of IBDV is hard to assess as it involves multi-factorial reasons. The loss has not only caused by the direct factor such as specific mortality (in acute form) but also involved indirect loss (immunosuppression). The latter conditions left the chicken susceptible to viral respiratory infections which result in increased mortality due to airsacculitis and colisepticemia. The loss also may occur due to growth retardations, rejection of carcasses showing signs of hemorrhage and

depression in egg productions (Müller *et al.*, 2003). Simulation on the projected lost due to the introduction of IBDV and their running cost had been made. In the first simulations, the introductions of IBDV in New Zealand will give estimates loss of about 10 million USD. The running costs also were estimated to increase up to 10% due to IBD infections (Christensen, 1985). In Nigeria, the economic loss due to IBDV infections in 3 year period starting from 2009 to 2011 were estimated to be over three billion Nigerian currency (Musa, I.W., Sai'du, L., and Abalaka, 2012).The economic impact caused by IBDV infections are great and can only prevented via vaccination, thus IBDV should be considered as one of the most important virus of poultry industries and their disease prevention should be prioritized.

2.4 PREVENTION AND CONTROL

IBDV had been reported to be stable and able to resist adverse environmental conditions and chemical treatments. They have been demonstrated to be stable at pH 3-9, resistant to heat (60°C, 1 hour), ether, chloroform, 1% SDS at 20°C, pH 7.5 for 30 min (Fauquet *et al.*, 2005) and ultraviolet light (Petek, D'Aprile, & Cancellotti, 1973). Even with thorough cleaning and disinfection, the virus can remain in the poultry houses for weeks (Lukert & Hitchner, 1984). So, the controls of infections by employing strict Biosafety programmed are useless against the outbreaks of the disease. The highly contagious property of the virus makes the matter worse. The only sufficient way to control the spread of the disease is through vaccinations. Before the spreads of vvIBDV, a combination of live and inactivated vaccines were suffice to prevent the infection, but with the emergence of the new pathotype, 'hotter' vaccines must be introduced. "Hotter" as a less attenuated virus need to be used to