UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF AN INDIRECT ELISA USING THE RECOMBINANT NUCLEOCAPSID PROTEIN AS ANTIGEN FOR DETECTION OF ANTIBODIES TO NEWCASTLE DISEASE VIRUS

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By

NORSHARINA BT MD SAAD

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

March 2007

To my dearest father and mother
for their infinite love, care and support.
I owe them everything I have today

To my beloved husband and son

Also my relatives and friends

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

Development of an Indirect Elisa Using the Recombinant Nucleocapsid Protein as Antigen for Detection of Antibodies to Newcastle Disease Virus

By

NORSHARINA BINTI MD SAAD

March 2007

Chairman : Professor Datin Paduka Khatijah Mohd. Yusoff, PhD

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Newcastle disease (ND) is a virulent form of poultry disease that has the potential to cause 100% mobidity and mortality. The continuing threat of ND to the poultry industry requires routine testing of vaccinated chickens to determine that they have been adequately immunised by vaccination. Enzyme-linked immunosorbent assays (ELISA) which use commercially available reagents are presented as alternatives to the procedure for detection of Newcastle disease virus (NDV) antibodies. The nucleocapsid protein has been shown to immunogenic in nature, and play key roles in diagnostic ELISA for detection of antibody.

A purified recombinant nucleocapsid protein (NP) of NDV highly expressed in *Escherichia coli* (*E. coli*) was used as the coating antigen in an indirect ELISA to detect the presence of NDV antibodies in a cohort of chicken sera. The test was standardised using sera from vaccination study and obtained from Charles River SPAFAS Laboratories, USA. The NP-ELISA was standardised at an antigen concentration of 1.5 μg/ml, test serum dilution of

1:200 and conjugate dilution of 1:2000. The cut-off value of the NP-ELISA of 0.238 was determined by a receiver operating characteristic (ROC) analysis. An assay of 315 chicken serum samples against NDV antibodies showed that the NP-ELISA had 92.06% sensitivity and 93.2% specificity compared to NDV hemagglutination–inhibition (HI) test. This was in good agreement between the two serological methods (κ =0.848). In the serum neutralization test (SNT), the result shows that there was no correlation observed between HI and SNT. It was observed that the field serum samples found positive or with high titer in the HI test were negative or have low titer for SNT. This suggests that different epitopes on the antigen have been recognized by each of the tests. The result shows that the recombinant NP antigen is recognized by antibodies specific to the other poultry pathogens and the cross reactivity is high for *Paramyxovirus-2* (*PMV-2*) and *Paramyxovirus-3* (*PMV-3*) but low reaction by other poultry pathogen [avian influenza (AI), avian encephalomyelitis (AE), infectious laryngotracheitis (ILT), infectious bronchitis (IB), and infectious bursal disease (IBD)]. The titre of ND antibodies can be determined using NP-ELISA formula, $Log_{10}Titre = 1.0* Log(S/P) + 3.45$. This formula was derived after getting the linearity graph between NP-ELISA to commercial ND test kit. This finding indicates the potential application of the *E. coli* produced NP protein as antigen in indirect ELISA for the detection of NDV antibody. The NP-ELISA was successfully developed and very useful which is more or as efficient as commercial kits, thus the cost of NP-ELISA is cheaper than commercial kits.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

Pembangunan Indirect Elisa menggunakan Protein Rekombinan Nukleokapsid sebagai Antigen untuk Mengesan Antibodi terhadap Virus Penyakit Newcastle

Oleh

NORSHARINA BINTI MD SAAD

Mac 2007

Pengerusi : Profesor Datin Paduka Khatijah Mohd. Yusoff, PhD

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Penyakit Newcastle adalah penyakit virus ternakan yang berpotensi menyebabkan 100% morbiditi dan kematian. Ancaman penyakit Newcastle yang berterusan berlaku dalam industri ternakan memerlukan ujian rutin bagi ayam yang telah divaksinkan, untuk menentukan ayam-ayam tersebut telah diimunisasi dengan mencukupi melalui vaksinasi. Produk ELISA yang telah dikomersilkan sekarang adalah sebagai kaedah alternatif dalam pengesanan antibodi virus penyakit Newcastle. Protein nucleocapsid telah menunjukkan immunogenik dalam keadaan semulajadi, dan berperanan dalam ujian diagnostik ELISA untuk mengesan antibody.

Protein rekombinan nuleocapsid daripada virus Newcastle ayam telah ditulenkan dan diekspreskan dengan banyaknya dalam sistem *E.coli* telah digunakan sebagai antigen dalam ujian ELISA untuk mengesan kehadiran antibodi virus Newcastle di dalam serum ayam. Ujian ini yang telah dipiawaikan menggunakan serum daripada kajian pemvaksinan dan diperolehi daripada makmal Charles River SPAFA, USA. Keadaan ujian NP-ELISA telah

dipiawaikan pada kepekatan antigen 1.5μg/mL, pencairan serum yang diuji pada 1:200 dan pencairan konjugat pada 1:2000. Nilai takat penentu NP-ELISA ialah 0.238 yang telah ditentukan melalui analisis ciri-ciri memproses penerimaan (ROC). Dalam menguji 315 sampel serum ayam terhadap antibodi virus Newcastle telah menunjukkan NP-ELISA mempunyai sensitiviti 92.06% dan spesifisiti 93.2% yang dibandingkan dengan ujian penghalang hemagglutinin (HI). Dalam dua ujian serologi ini menunjukkan persetujuan yang baik dengan nilai $\kappa = 0.848$. Dalam ujian peneutralan serum, keputusan menunjukkan tiada hubungan antara HI dan ujian peneutralan tersebut. Ini telah diuji apabila sampel serum lapangan menunjukkan bacaan titer yang tinggi dengan ujian HI tetapi sebaliknya bagi ujian peneutralan serum. Setiap ujian akan mengenali antibodi yang berbeza berdasarkan epitop pada antigen dalam ujian yang digunakan. Keputusan ujian menunjukkan antigen rekombinan NP dapat dikenali oleh antibodi patogen ternakan yang lain dan tindak balas bercanggah yang tinggi berlaku pada *PMV-2* dan *PMV-3*, tetapi tindak balas bercanggah yang rendah pada patogen ternakan yang lain [avian influenza (AI), avian encephalomyelitis (AE), infectious laryngotracheitis (ILT), infectious bronchitis (IB), and infectious bursal disease (IBD)]. Titer antibodi bagi penyakit Newcastle boleh ditentukan dengan menggunakan formula NP-ELISA, $Log_{10}Titer=1.0*Log\ (S/P)+3.45$. Formula ini ditentukan apabila mendapatkan graf yang linear antara NP-ELISA dengan produk ELISA komersial.. Penemuan ini menunjukkan potensi kegunaan protein NP yang dihasilkan dalam E.coli sebagai antigen dalam 'indirect' ELISA untuk mengesan antibodi virus Newcastle. NP-ELISA telah berjaya dihasilkan dan sangat berguna di mana ianya adalah sama atau lebih efisen dengan produk komersial. Tambahan pula kos NP-ELISA adalah murah daripada produk komersial.

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I certify that an Examination Committee met on 23rd March 2007 to conduct the final examination of Norsharina Bt Md Saad on her Master of Science thesis entitled "**Development of an indirect ELISA using the recombinant nucleocapsid protein as antigen for detection of antibodies to Newcastle Disease Virus**" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree.

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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(NORSHARINA BT MD SAAD)

Date: 21 May 2007

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AE	avian encephalomyelitis
AI	avian influenza
APMV-1	avian paramyxovirus type-1
APMV-2	avian paramyxovirus type-2
APS	ammonium persulfate
BCIP	bromochloroindolyl phosphate
BSA	bovine serum albumin
CEF	chicken embryo fibroblast
CPE	cytophatic effect
dH_2O	distilled water
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EID_{50}	50% egg infectious dose
ELISA	enzyme-linked immunosorbent assay
F	fusion (protein)
g	gravity
h	hour
HA	haemagglutinin activity or haemagglutination
HAU	haemagglutination unit
HI	haemagglutination inhibition
ICPI	intracerebral pathogenicity index
ICTV	International Committee on the Taxonomy of Viruses

Ig	immunoglobulin
IB	infectious bronchitis
IBD	infectious bursal disease
ILT	infectious laryngotracheitis
IPTG	isopropyl-β--thiogalactopyranoside
IVPI	intravenous pathogenicity index
kb	kilobase
L	large (protein)
LB	Lubria-Bertani medium
Log	logarithma
M	matrix (protein)
mAb	monoclonal antibody
MDT	mean death time
min	minute
mRNA	messenger ribonucleic acid
NA	neuraminidase activity
NBT	nitro blue tetrazolium
ND	Newcastle disease
NDV	Newcastle disease virus
NI	neuraminidase inhibition
NP	nucleocapsid (protein)
NusA	N-utilising substance A (protein)
OD_x	optical density (or absorbance) at x nm

OIE	Office of International des Epizootics
OPD	*o-phenylenediamine*
P	phosphoprotein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffer saline
PET	polyethylene tube
RBC	red blood cell
rpm	Revolution per minute
RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
SNT	serum neutralization test
SPF	specific pathogen-free
$TCID_{50}$	50% tissue culture infectious dose
TEMED	N,N,N'N'-tetramethyethylenediamine
TMB	3,3',5,5',-tetramethylbenzidine
TPBS	Tween 20- phosphate buffer saline
VN	virus neutralization
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

Introduction

Newcastle disease (ND) is a widespread and an economically important poultry disease which affects chickens, turkeys and others birds. As a result, our poultry industries suffer huge losses with high mortality, poor food utilization and reduced egg production. It is caused by the Newcastle disease virus (NDV). The virus not only attacks poultry and birds, but it also infects man and other mammals. Unlike in the other animals, ND has been recognized in man almost entirely as localized eye infection (Brandly and Hanson, 1965), headache, and flu-like symptoms rarely becoming severe or leading to visual impairment.

The virus is relatively stable in nature, remaining infective for weeks at room temperature (22°C) and surviving for several hours in a wide range of pH (3-10). It can survive for 20 days in the forest litter but the survival can be prolonged to 255 days in water, soil, carcasses, eggs and feathers (Hanson, 1980). The survival rate can be prolonged even further in meat and bones up to 6 months at 1°C. On the other hand, the virus can be inactivated by pasteurization of eggs and egg products at 64°C for 4.5 minutes, processing meat for 30 minutes at 30°C or 1 minute at 80°C. Transmission is by contact with infected products or by aerosol from infected live birds. Mechanical transmission between premises can occur from contaminated footwear, clothing, skin, feed, trucks, and poultry or egg handling equipment.

NDV in its many different forms and pathotypes occurs as regular, frequent epizootics throughout Asia, Africa, and South America, and sporadic epizootics occur in Europe (Williams *et al*., 1997) (**Figure 1**; OIE 2002). The virus is enzootic in some areas of the world including Malaysia where sporadic outbreaks occur. As reported by the Veterinary Research Institute, Ipoh, 38 new cases were isolated from chickens in 2003. However, in 2004 the NDV cases increased to 122 cases; majority of these were isolated from chickens with four cases from ducks. The number of cases increased to 130 (from chicken) and 16 (from duck) in 2005. However, in 2006, the cases of NDV were reduced to 107 and 5 cases isolated from chicken and duck respectively. Most of the domestic outbreaks occurred in areas with high density of poultry farms, with reduced number of outbreaks among small holders and in the village.

To date, vaccination by coarse spray is seldom practised in Malaysia. The instructions given in the vaccine manufacturer's pamphlet for the particular vaccine should be studied by the farmers carefully in terms of storage, handling, dilution and vaccination routes. Efforts to raise the awareness of farmers and the general public of the disease and current control measures have been very effective judging by the response to the call for vaccination.

In addition, efforts to control and prevent ND through efficient vaccination programs and corresponding serological monitoring are also constantly being carried out (Ricardo *et al*., 2000). The continuing threat of ND to the poultry industry requires routine testing of vaccinated chickens to determine that they have been adequately immunized by vaccination. Diagnosis of ND has progressed from the conventional virus isolation and serological tests to the use of monoclonal antibodies and semi-automated enzyme

linked immunoassay (ELISA). These are further improved by the application of molecular-based techniques, such as reverse transcription polymerase chain reaction (RT-PCR), oligonucleotide probes, genomic fingerprint and nucleotide sequencing (reviewed in Aldous and Alexander, 2001; Yusoff and Tan, 2001). The choice of the diagnostic methods is mainly based on the consideration of cost, speed, specificity and sample size.

The haemagglutination-inhibition (HI) test is the most widely used conventional serological method for detection of NDV antibodies as it is quite specific and gives reproducible results (Ricardo *et al*., 2000). In recent years, various indirect ELISAs have been developed and evaluated (Snyder *et al*., 1983; Thayer *et al*., 1987; Adair *et al*., 1989; de Witt *et al*., 1992), and these have been correlated to the HI test (Brown *et al*., 1990). The potential applications of the recombinant NP protein as alternative antigen in indirect ELISA to detect the presence of ND antibodies, and in diagnosis of ND were explored in this study as they could contribute to the screening and monitoring of the disease.

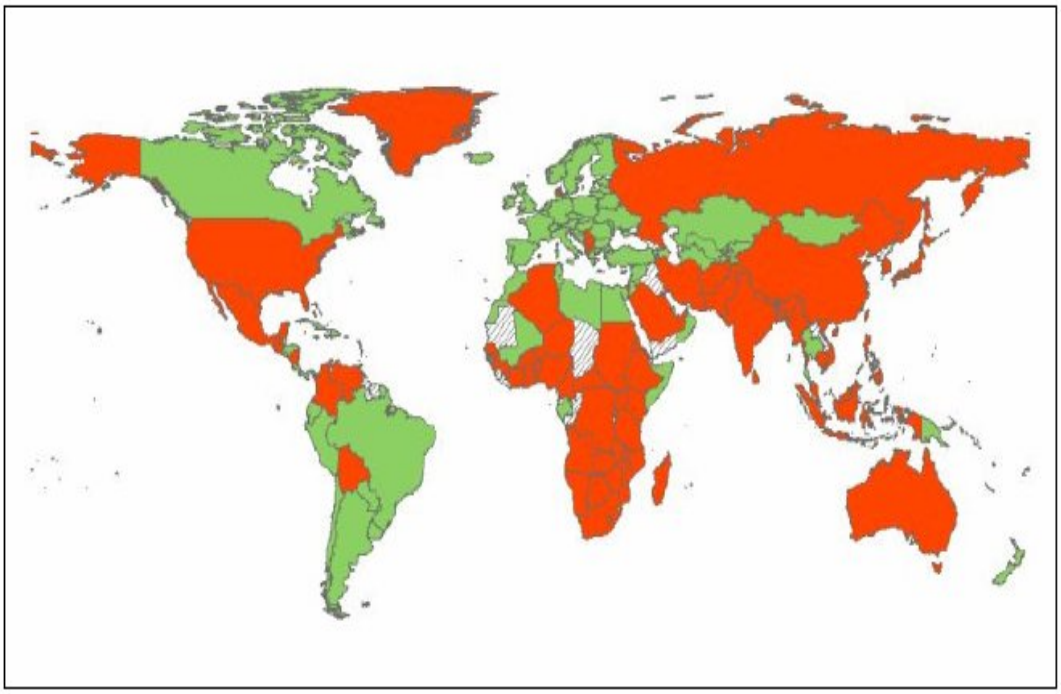

Figure 1: Animal Health Status Worldwide in 2002 (Source: OIE, Animal Health Status Worldwide in 2002). Note that due to rigorous vaccination programmes ND is no longer endemic in many countries (OIE., Animal Health Status Worldwide in 2005)

Disease reported present

Disease reported absent

Data unavailable or incomplete

Significance of the study

The objective of this study was to produce the recombinant NP protein in bulk as antigen for indirect ELISA to detect the presence of anti-NDV antibodies. It is also aimed to study the sensitivity and specificity of the NP-ELISA using true positive and negative sera. Furthermore, the NP-ELISA was also compared with the HI test, serum neutralization test (SNT) and other ELISA tests. It is also important to ensure production of such an NP-ELISA test kit is as efficient as the other commercially available kits such as Idexx and BioChek are usually very expensive, whereas a locally produced NP-ELISA test kit can reduce the cost of ND testing for screening in surveillance and monitoring programmes.