UNIVERSITI PUTRA MALAYSIA

PEPTIDE LIGANDS THAT INTERACT WITH NEWCASTLE DISEASE VIRUS:SELECTION, CHARACTERIZATION AND APPLICATIONS

PRIADARISHNI RAMANUJAM-THURAI RAJA

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By

PRIADARISHNI RAMANUJAM-THURAI RAJA

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

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DEDICATION

TO MY BELOVED FATHER,

WHO WILL ALWAYS BE WITH ME AND WHO WAS THE INSPIRATION AND MOTIVATION FOR ME TO DO MY PHD

TO MY DEAREST MOTHER,

WHO IS ALWAYS PRAYING FOR ME, SOMEONE WHO GAVE ME CONFIDENCE AND STOOD BY ME THROUHGOUT THE STUDY

TO MY DARLING HUSBAND,

WHO IS ALWAYS THERE FOR ME, WHO HAS MADE MYRIAD SACRIFICES AND WAS THE STRENGTH FOR ME TO COMPLETE THIS STUDY

TO MY MOST ADORED NEPHEWS AND NIECES,

Vel, Mumu, Shara, Nana, Meha, Shankar (K.Kutty), Lavin (C. Noyanram), Logan, Gaiyan (Champion), Yoyo (MM), Keshu, Din (Sayang), Umani (Kuchu) and Uthi Boy (Simba)

FOR THEIR ENTHUSIASM IN VISITING ME IN MY LAB AND MAKING ME FEEL LIKE THE BEST SCIENTIST EVER

I DEDICATE THIS TO ALL OF YOU...

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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PRIADARISHNI RAMANUJAM-THURAI RAJA

January 2003

Chairperson: Assoc. Prof. Dr. Tan Wen Siang

Faculty: Science and Environmental Studies

Newcastle disease, a highly contagious disease of the avian species is caused by the Newcastle disease virus (NDV). In Malaysia there have been 29 outbreaks of NDV reported this year alone. In spite of the availability of mass vaccination programmes, these sporadic outbreaks are believed to be the result of malpractice in backyard farming. The mechanism of NDV infection is still not clearly understood, making it difficult to validate any effective drug candidates. Bearing this in mind, a phage display random peptide library was used to identify peptide ligands that interact with NDV. A phage carrying the TLTTKLY sequence represented 97% of the total phage screened from the third round of biopanning against NDV strain AF2240. This phage was characterized and both cyclic and linear synthetic peptides bearing the homologous sequence were synthesized.

The phage was able to compete with the polyclonal antibodies raised against AF2240 for binding sites on the virus. Furthermore, the synthetic peptides proved to be stronger binders to AF2240 than the TLTTKLY phage. The synthetic peptides did not inhibit the haemagglutination and the neuraminidase activities of the virus, but it reduced the viral haemolytic activity, suggesting that the peptide binds at a 'biologically active site' on the virus. In addition, the synthetic peptides inhibited the propagation of the virus in embryonated chicken eggs, with IC_{50} values of 5 and 10 μM for the cyclic and linear peptides respectively. This signifies its potential role as a precursor for drug candidate.

The relative dissociation constant curve showed that there are two binding sites for the TLTTKLY phage with various NDV strains. All the strains displayed low K_d^{rel} values with the first K_d^{rel} ranging from 2.4 to 4.0 pM and the second K_d^{rel} in the nanomolar range. The TLTTKLY phage was useful not only as a detecting agent of NDV, but also for pathotyping the NDV strains. The TLTTKLY phage can be used to distinguish the velogenic strain from the mesogenic and the lentogenic strains, which is in line with the first K_d^{rel} obtained. With that, it is hoped that the TLTTKLY phage could represent a novel drug design or functions as a useful diagnostic reagent for NDV.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

LIGAN PEPTIDA YANG BERINTERAKSI DENGAN VIRUS PENYAKIT NEWCASTLE: PEMILIHAN, PENCIRIAN DAN KEGUNAAN

Oleh

PRIADARISHNI RAMANUJAM-THURAI RAJA

January 2003

Pengerusi: Prof. Madya Dr. Tan Wen Siang

Fakulti: Sains dan Pengajian Alam Sekitar

Penyakit Newcastle adalah suatu penyakit berjangkit dalam spesies burung yang disebabkan oleh virus Newcastle disease (NDV). Di Malaysia, terdapat 29 kes kejadian yang telah dilaporkan pada tahun ini sahaja. Walaupun terdapat program vaksinasi, namun begitu masih banyak kejadian dilaporkan yang berkemungkinan disebabkan oleh penyalah-laksanaan amalan penternakan. Mekanisme infeksi NDV masih kurang jelas dan ini menyukarkan penghasilan calon ubat yang berkesan. Disebabkan perkara ini, suatu perpustakaan persembahan faj digunakan untuk mengenalpasti ligan peptida yang boleh berinteraksi dengan NDV. Faj yang membawa jujukan asid amino TLTTKLY meliputi 97% daripada jumlah faj yang disaring daripada pusingan ketiga pemilihan berafiniti dengan NDV strain AF2240. Pencirian faj telah dilaksanakan dan peptida berkonformasi dan tidak berkonformasi sintetik yang homologi dengan faj telah disintesis.

Faj ini didapati mampu bersaing dengan antibodi poliklon yang dihasilkan terhadap AF2240 untuk tapak pengikatan pada virus tersebut. Tambahan lagi, peptida sintetik ini menunjukkan kekuatan interaksi yang lebih kuat terhadap AF2240 berbanding dengan faj TLTTKLY. Peptida sintetik tersebut tidak merencat aktiviti hemaglutinasi dan neuraminidase NDV, tetapi ia mengurangkan aktiviti hemolitik virus tersebut. Ini menunjukkan bahawa peptida mungkin terikat pada 'tapak aktif biologi' virus. Di samping itu, peptida sintetik merencat pertumbuhan virus ini dalam telur ayam berembrio, dengan nilai IC_{50} 5 dan 10 μM masing-masing, bagi peptida berkonformasi dan peptida tidak berkonformasi. Ini mengambarkan peranan peptida tersebut sebagai pelapor calon perubatan.

Lekuk pemalar penguraian relatif menunjukkan terdapat dua tapak pengikatan di antara faj TLTTKLY dengan pelbagai strain NDV. Kesemua strain tersebut memaparkan nilai K_d^{rel} yang rendah dengan K_d^{rel} pertama di antara 2.4 dan 4.0 pM manakala K_d^{rel} kedua dalam julat nanomolar. Faj TLTTKLY berguna bukan sekadar untuk mengenalpasti kehadiran NDV tetapi juga sebagai agen membezakan patotaip strain NDV. Faj TLTTKLY boleh digunakan untuk membeza strain velogenik daripada strain mesogenik dan strain lentogenik, yang sejajar dengan K_d^{rel} pertama yang diperolehi. Dengan ini, adalah diharapkan, faj TLTTKLY boleh mewakili rekaan dadah baru atau sebagai reagen diagnostik untuk NDV.

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I certify that an Examination Committee met on 16th January 2003 to conduct the final examination of Priadarishni Ramanujam on her Doctor of Philosophy thesis entitled "Peptide Ligands that Interact with New Castle Desease Virus: Selection Characterization and Applications" in accordance with the 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. Members of the Examination Committee are as:

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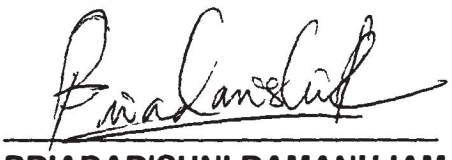
DECLARATION

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.



PRIADARISHNI RAMANUJAM

Date: 10/02/03

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LIST OF ABBREVIATIONS

A_{600}	absorbance at wavelength 600 nm
α	alpha
APS	ammonium persulfate
β	beta
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
C-terminus	carboxyl terminus
DEPC	diethyl pyrocarbonate
dNTP	deoxyribonucleotide phosphate
DNA	deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
gp3 & gp8	products of M13 genes 3 and 8, respectively
HA	haemagglutination activity
HIV	human immunodeficiency virus
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
K_d^{rel}	relative dissociation constant
λ	lambda
LB	Luria broth
M	molar
mAb	monoclonal antibody
mM	millimolar
mRNA	messenger RNA
NA	neuraminidase activity
ND	Newcastle disease
NDV	Newcastle disease virus
nm	nanometer (10^{-9} m)
N-terminus	amino-terminus
OD	optical density
pAb	polyclonal antibody
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PEG	polyethylene glycol
pfu	plaque forming unit
pM	picomolar
QLGGPSH phage	phage carrying the QLGGPSH fusion peptide
RE	restriction enzyme
RNA	ribonucleic acid

RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyle sulphate-polyacrylamide gel electrophoresis
TBS	tris-buffered saline
TE	tris-EDTA buffer
TEMED	tetramethyl ethylenediamine
TLTTKLY phage	phage carrying the TLTTKLY fusion peptide
U	unit (s)
UV	ultraviolet
vol	volume
v/v	volume/volume
W	watt
w/v	weight/volume
xg	centrifugal force
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
%	percentage
°C	degree centigrade

CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is a highly contagious viral disease of domestic poultry and it remains as a major impediment to village poultry production. This disease not only affects domestic fowls, but also turkeys, pheasants, pigeons, quails and guinea fowls to a varying degree. Ducks and geese are also susceptible to infection, but these species rarely succumb to the disease. ND has been classified as the List A disease under the *Office International des Epizooties* (OIE), which is termed as, "*Transmissible diseases that have the potential for very serious and rapid spread, irrespective of national borders, that are of serious socio-economic or public health consequence and that are of major importance in the international trade of animals and animal products*".

All age groups of avian species are susceptible to ND infection. Newcastle disease virus (NDV) strains are classified into 3 groups related to the severity of the disease (Beaudette & Black, 1946). Velogenic strains cause sudden onset of depression, rapid breathing and anorexia in birds and a sharp drop in egg production in laying hens. The viscerotropic velogenic strains may cause edema of the head and diarrhoea as a prominent clinical feature whilst, the neurotropic velogenic strain are distinguished by marked nervous signs such as torticollis, tremors or paralysis (Purchase *et al.*, 1989). The mesogenic

strains cause respiratory and sometimes nervous signs with low mortality whereas; in the lentogenic strains there is a mild or imperceptible respiratory infection (Jordan, 1990).

NDV is endemic in many countries, particularly in Asia, the Middle East, Africa and Central and South America. Some European countries are considered free of NDV, although this disease has caused high mortality in wild cormorants in Canada and the United States. Throughout the world the number of outbreaks recorded for the years 2001, 2000 and 1999 were approximately 3200, 2930 and 2240 cases respectively and for Asia alone, there were 1670, 1460 and 1410 cases, respectively. A recent epidemic in Southern Jutland County, Denmark (August 2002) revealed that, 110 of the 118 verified cases were detected in backyard fowls. There were confirmed deaths of birds between 500-1000 cases over 4-6 weeks and up to a 40% drop in egg production in Victoria, Australia when an ND incidence emerged in May 2002 (OIE homepage, http://www.oie.int.). Eighty-five deaths were cited at a cockfight centre in Valera district, State of Trujillo, Venezuela due to ND occurrence. Zulia, another district in Venezuela experienced a total of 5 outbreaks with 85,000 deaths reported when an outbreak took place in June 2002. The prevalence in Chiba Prefecture, Japan in June 2002 affected the population of young pheasants, which caused a total number of 380 deaths. In Sudan (Al Kalakla area and Al Geraif area in Khartoum), 2 epidemics that emerged caused 1003 deaths (OIE homepage, http://www.oie.int.).

In Malaysia, there were approximately 52,500 deaths owing to 96 outbreaks reported in the year 2001. Despite the fact that the number of deaths was reduced (367,218) compared to the year 2000, the number of outbreaks was nevertheless higher in 2001. Until the month of March 2002, there were a total of 29 cases of epidemic involving well over 41,000 infected avian species (OIE homepage, http://www.oie.int.).

These figures render an urgent need to eradicate this disease, but it is however an intricate task to accomplish, as NDV has a wide range of hosts and it is easily transmittable. In addition, the virus remains infective for several weeks at low temperature, surviving for several hours in a wide range of pH (3-10) and for about 225 days in water, soil, carcasses, eggs and feathers. It is no doubt that vaccination with live and/or oil emulsion vaccines have markedly reduced the losses in the poultry flocks but hitherto, there is no treatment for this disease.

To find a cure for this disease, the exact mechanism of NDV infection in the host cell should be well understood. The lack of information of the viral infection mechanism has caused us millions of avian lives. To understand the interaction between NDV and its host cell, a phage displayed random peptide library was therefore used to select for peptide ligands that interact with this virus.

A phage display 'library' is a heterogeneous mixture of such fusion phage clones, each carrying a different foreign DNA insert and therefore displaying a different peptide on its surface (Smith & Petrenko, 1997). Over the past decade, phage display has been the subject of intensive research whereby it represents not only a powerful technology for selecting but also for engineering polypeptides with novel functions. As a result, phage display and its modifications have become the practical tools for drug discovery, phage derived antibody libraries, selection of enzyme inhibitors, ligands for intracellular protein-binding domains, mimics for extracellular protein-protein interactions and much more.

The display of a foreign peptide was first demonstrated by Smith (1985), when he inserted a Sau 3A digest of plasmid pAN4 at the gene III insertion site of a filamentous bacteriophage. It was found that the inserted gene was expressed as a fusion protein in the middle with the gpIII protein of the phage. Most importantly, the recombinant phage retained its infectivity and the displayed foreign amino acids were in their immunologically accessible form. This discovery set a platform for what has been achieved with this renowned technology to date.

Over the years, methods have been refined to produce libraries of high diversity and great quality as the success of a selection experiment ultimately depends on these. An improved technique of library construction with diversities greater than 10^{12} different recombinant fusion phage was described by Sidhu *et*

al. (2000). In the initial paradigm, polypeptides were either fused to the amino-terminus of the coat protein p3 or p8 of the viral genome, which severely limited large polypeptides to be efficiently displayed. However with the development of phagemid display systems, this problem was solved. Here, polypeptides were fused to an additional coat protein gene encoded by a phagemid vector (Bass e*t al*., 1990).

The phagemid system has enabled the development of new display systems. Proteins can be displayed as fusions to the carboxyl-terminus of p6 protein (Jespers e*t al*., 1995) and also p8 protein (Fuh e*t al*., 2000). Gao e*t al*. (1999) demonstrated the display of antibody fragments fused to the amino-terminus of p7 and p9 proteins. It was also noted that the level of display of polypeptides at the carboxyl-terminus is on par with the conventional amino-terminus fusion (Fuh & Sidhu, 2000). Thus, in phagemid systems, functional polypeptides have been demonstrated with all five M13 coat proteins.

The revolution of phage display technology induced a great interest and curiosity in us. Since the peptide ligands that interact with NDV were not conclusively determined, we exercised to solve this task by applying a disulfide constrained phage display random peptide library to select for ligands to NDV. Thus, on the basis of that thrust, the objectives of this study are as follows:

1. Select for peptide ligands that interact with NDV;

2. Characterize the selected peptide sequences; and

3. Study the applications of the selected phage and synthetic peptides.

CHAPTER 2

LITERATURE REVIEW

2.1 Newcastle disease

2.1.1 Historical aspect

In the spring of 1926, on a farm near Newcastle-upon-Tyne, England, the first outbreak of a highly pathogenic virus occurred. Doyle (1927) named this emergence as Newcastle disease (ND), and it was believed that there was a possible link between this and the outbreak in Java, Indonesia in March 1926. It has been considered that the presence of the virus in England was the result of transportation of frozen meat and live chickens from Southeast Asia to Newcastle-upon-Tyne by ship (Alexander, 1988).

Generally, Asia has been regarded as the native land for ND as the report of the first outbreak came from Java in 1926. Furthermore, since 1982, ND has been endemic throughout Southeast Asia (Shortridge, 1982). In Malaysia, a velogenic strain was isolated in a field outbreak in the 1960s (Lai & Ibrahim, 1987). This strain was called AF2240 and it caused a very high morbidity and mortality rate among the poultry flocks. This disease remains endemic in many regions and continues to severely limit the poultry production. It is particularly