UNIVERSITI PUTRA MALAYSIA

PATHOGENICITY AND CHARACTERISATION OF NEWCASTLE DISEASE VIRUS ISOLATED IN IRAN

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By

MAHDI KIANIZADEH

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This Thesis is dedicated especially to

My wife and children

Azam Sobhani

and

Hamid

Farzaneh

Hamed

Who have given me strength and courage with their patience

To carry-out this program of research.

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Chairperson : **Professor Aini Ideris, Ph.D.**

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Nine Iranian Iranian Newcastle disease viruses were isolated from different Newcastle disease outbreaks during 1995 to 1999 across Iran. Characterization of the isolates was performed using three standard conventional methods such as mean death time (MDT), intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) in embryonated specific pathogen free (SPF) chicken eggs, day old SPF chicks and 6–week old SPF chickens, respectively. Based on the overall results, all the nine Iranian isolates (MK7, MK12, MK13, MK14, Krd76, Kasra97, GH77, KH2/78 and ES1/99) belong to velogenic pathotype.

Following infection in 6-week old SPF chickens via intra-ocular route, regardless of the isolates, the infected chickens showed depression, ruffled feather and diarrhoea, ending in death. However, there were no obvious respiratory disorders. Meanwhile nervous signs were shown in chickens

inoculated with four isolates (MK7, GH77, MK13 and Kasra97) at the later stage of the infection. Grossly, the initial lesions such as haemorrhage and enlargement were observed in visceral organs including proventriculus, intestine and spleen. No significant changes were observed in the respiratory system. However in some cases, chickens infected with some isolates (MK7, MK12, MK13, Kasra97, KH2/78 and ES1/99) demonstrated limited congestion in the trachea and lungs. Microscopically, the earliest changes were observed in proventriculus, intestine, spleen and liver. Focal and diffuse haemorrhage (proventriculus and intestine), lymphoid depletion (spleen) and mononuclear cell infiltration (liver) were common features. Endotheliosis and malacia were seen in the brain. Based on the MDT results (39.6-56 hours) as well as the presence of lesions in the visceral organs, all the isolates can be grouped as viscerotrop velogenic Newcastle disease virus (VVNDV) pathotype.

The F protein cleavage site gene of all the nine isolates was amplified (242 bp) by RT-PCR and sequenced. Nucleotide and amino acid sequence analysis revealed that the cleavage site of all the isolates consisted of 2 pairs of basic amino acids (Arg) at positions 112,113, 115 and 116 forming the motif ^{112}RRQRR^{116}, similar to motif in velogenic strains isolated from other countries. However, five amino acids substitutions were observed in isolate MK13 compared to other isolates of which 2 were in F_1 N-terminal, 2 upstream and one downstream of the cleavage site. Two of the amino acids differences in MK13 located at positions 108 and 132 were not observed in published sequence of NDV strains.

Hydropathy profiles were similar for all the isolates. They contained one major hydrophilic area at cleavage site and one major hydrophobic area extended between positions 117 to 142 corresponded to the F_1 N- terminal. This feature of hydropathy profile is similar to that of virulent NDV strains. In phylogenetic analysis, all Iranian isolates are grouped together with other virulent strains in group C. The Russian virulent strain, VOL 95, showed the same amino acid sequence with all the Iranian isolates except MK13 indicating a common origin of the viruses. The importance of these substitution remain to be studied. Finally, based on the results obtained, this study proved that all nine Iranian NDV isolates belonged to the velogenic pathotype.

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KEPATOGENAN DAN PENCIRIAN ISOLAT VIRUS PENYAKIT SAMPAR AYAM DARIPADA IRAN

Oleh

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Januari 2002

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Sembilan virus tempatan penyakit sampar Iran dipencilkan dari beberapa wabak penyakit sampar ayam di Iran dari tahun 1995 hingga 1999. Pencirian setiap isolat dijalankan berdasakan tiga kaedah konvensional seperti MDT (Mean deat time), ICPI (Intracerebral pathogenicity index) dan IVPI di (Intravenous pathogenicity index) dalam telur ayam SPF (specific pathogen free) berembrio, anak ayam SPF berusia satu hari dan ayam SPF berusia 6 minggu. Berdasarkan keputusan keseluruhan yang didapati, kesemua sembilan isolat tempatan tersebut (MK7, MK12, MK13, MK14, Krd76, Kasra97, GH77, KH2/78 dan ES1/99) digolongkan dalam patotip velogenik.

Berikutan daripada jangkitan pada ayam SPF berusia 6 minggu dengan kesemua isolat, melalui cara intra-okular, ayam yang dijangkiti menunjukkan kemurungan, bulu berserabut dan cirit-birit yang membawa kepada kematian. Walau bagaimanapun tiada masalah pernafasan yang ketara. Manakala, tanda kegugupan yang disebabkan oleh beberapa isolat (MK7, GH77, MK13 dan Kasra97) dapat dilihat pada fasa akhir jangkitan. Secara

GH77, MK13 dan Kasra97) dapat dilihat pada fasa akhir jangkitan. Secara kasar, lesi awal seperti hemoraj dan pembesaran organ dapat dilihat pada organ visera termasuk proventrikulus, usus dan limpa. Sistem pernafasan tidak menunjukkan sebarang lesi yang ketara. Walau bagaimanapun, dalam setengah kes ayam yang dijangkiti oleh beberapa isolat (MK7, MK12, MK13, Kasra97, KH2/78 dan ES1/99) menunjukkan sedikit kesebakan pada trakea dan paru-paru. Secara mikroskopik, perubahan awal dapat dilihat pada proventrikulus, usus, limpa dan hati. Hemoraj setempat dan tersebar (proventrikulus dan usus), kemusnahan limfoid (limpa) dan infiltrasi sel mononuklear (hati) merupakan cirri-ciri utama. Endoteliosis dan malasia diperhatikan pada bahagian otak. Berdasarkan keputusan MDT (39.6-56 jam) dan juga kehadiran lesi di organ visera, kesemua isolat tempatan tersebut dikategorikan sebagai VVNDV.

Gen tapak belahan protein F bagi semua isolat diamplifikasi (242bp) melalui RT-PCR dan dijujuk. Analysis jujukan nukleotida dan asid amino mendedahkan bahawa tapak belahan bagi kesemua isolat terdiri daripada 2 pasang asid amino basik (Arg) pada kedudukan 112, 113, 115 dan 116 yang membentuk motif ^{112}RRQRR^{116}, menyerupai motif strain velogenik yang dipencil dari negara lain. Walau bagaimanapun, penggantian lima asid amino diperhatikan dalam isolat MK13 berbanding kepada isolat lain yang mana 2 pada terminus-N F1, 2 di hadapan dan 1 dibelakang daripada tapak belahan. Perbezaan dalam 2 asid amino pada MK13 yang terletak pada kedudukan 108 dan 132 tidak diperhatikan dalam jujukan strain NDV yang pernah diterbitkan. Kepentingan penukaran asid amino ini perlu dibuat kajian.

Profil hidropati bagi kesemua isolat adalah serupa. Mereka mempunyai satu kawasan hidrofilik major pada tapak belahan dan satu kawasan hidrofobik major yang menganjur dari kedudukan di antara 117 hingga 142 yang berdekatan dengan terminus-N F1. Ciri profil hidropati ini adalah sama dengan strain NDV virulen. Dalam analisis filogen, kesemua isolat tempatan digolongkan bersama beberapa strain virulent yang lain dalam kumpulan C. Strain virulen Rusia, VOL95, menunjukkan jujukan asid amino yang sama dengan kesemua isolat tempatan kecuali MK13 menandakan persamaan asal-usul bagi virus-virus tersebut. Berdasarkan keputusan yang diperolehi, kajian ini membuktikan bahawa kesemua sembilan isolat NDV Iran tergolong dalam patotip velogenik.

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TABLE OF CONTENTS

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

A_{260}	Absorption at 260 nm
AI	Avian Influenza
Amp	Ampicillin
Arg	Argenine
bp	Base pairs
CAM	Chorio allantoic membrane
CEC	Council of the economic community
CEF	Chicken embryo fibroblast
CPE	Cytopathic effect
CNS	Central nervous system
Da	Dalton
DEPC	Diethyl pyrocarbonate
DNA	Deoxynucleotide acid
dNTP	Deoxynucleotide triphosphate
ddNTP	Dideoxynucleotide triphosphate
dpi	Days post infection
EDTA	Ethylene diamine tetra acetate
EID	Embryo infection dose
EtOH	Ethanol
ELISA	Enzyme linked immunosorbent assay
F	Fusion protein
GIT	Gastro intestinal tract
HA	Haemagglutinin activity
HI	Haemagglutinin inhibition
HN	Haemagglutinin neuraminidase
hpi	Hours post infection
ICPI	Intra cerebral pathogenicity index
IPTG	Isopropyl-thiogalactopyranoside
IVPI	Intra venous pathogenicity index
Kb	kilo base
KD	kilo dalton
L	Large protein
LB	Luria-bertani
M	Membrane protein
Mabs	Monoclonal antibody
MCS	Multi cloning site
MLD	Minimum lethal dose
mRNA	Messenger RNA
nm	Nanometer
NP	Nucleoprotein
NTE	Natrium chloride-tris-EDTA buffer
OIE	Office International Des Epizooties
ORF	Open reading frame
OD	Optical density

P	Phosphoprotein
Pmol	Picomol
RNA	Ribose nucleic acid
RNase	Ribonuclease
RBCs	Red Blood Cells
SPF	Specific- Pathogen- Free
TAE	Tris-acetate-EDTA buffer
Taq	Thermus aquaticus
V	Valine
VVNDV	Viscerotropic velogenic Newcastle disease virus
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

CHAPTER I

INTRODUCTION

In the past decades, the systems of poultry husbandry have changed greatly, from small units of birds which were allowed to range freely in fields, to the systems of very large-scale rearing in "broiler house" in which birds are reared intensively or in battery cages for layers. A broiler house may contain as many as 100,000 chickens and there may be a quarter of a million birds on one site. Breeding stocks are also kept intensively, in houses with deep litter.

Keeping such large numbers of birds in close proximity that is less coordinate with physiological system of poultry, adds greatly to the economic loss resulting from a rapid spreading of viral infection of the respiratory system. The control of disease among poultry is further complicated by the present easy transportation of birds and eggs. Among the respiratory diseases, Newcastle disease (ND) has been the major problem in the poultry industry.

Newcastle disease is an infectious ailment of poultry that manifests itself in severe respiratory and nervous conditions. Its causative agent is paramyxovirus, a highly contagious pathogen (Wehmann *et al*., 1997), known as Newcastle disease virus (NDV). It is an enveloped virus and has a non-segmented negative single stranded RNA genome of approximately 15 kb which codes for six major proteins (Seal *et al*., 1995).

Outbreaks of ND were first reported in poultry in Java, Indonesia and Newcastle-Upon-Tyne, England in 1926 (Alexander, 1988). From the literature, three panzootics of ND could be recognized. The first appeared in South East Asia in 1926 and spread to most countries of the world. The second panzootic began in the Middle-East in the late 1960s and spread quickly to the other countries by 1973. The third panzootic caused by neurotropic form of ND apparently also started in the Middle-East in the late 1970s, reached Europe by 1981 and spread rapidly worldwide (Alexander, 1988). The disease, currently, has a worldwide distribution with a wide host range in which all orders of birds have been reported to be infected by Newcastle disease virus (NDV). The infectious virus may be ingested or inhaled spreading from one bird to another. This is its major form of transmission and is the basis for mass application of live vaccines via the spray or drinking route (Alexander, 1991).

Isolates of NDV are categorized into three main pathotypes depending on the severity of the disease produced by the isolates in chickens. Lentogenic isolates do not usually cause any disease in adult birds and are considered avirulent. Viruses of intermediate virulence that cause respiratory disease are termed mesogenic while virulent viruses that cause high mortality are termed velogenic. Neurotropic and viscerotropic forms of velogenic viruses have been reported worldwide (Seal *et al.*, 1995).

ND has now been with poultry industry for more than 70 years. Though vaccination for ND has been used successfully from the 1940s (Beard and

Hanson, 1987), its potential to cause great losses in the global poultry industry is probably going to continue as long as this virus can survive and replicate.

In routine diagnosis accessions, NDV isolates are readily identified by haemagglutination-inhibition (HI) with NDV antiserum, and pathogenicity study is often limited to determination of embryo death time (King and Seal, 1998). From the first report of the disease to date, many efforts have been made to develop methods to identify the NDV isolates and to determine their properties. Despite the large number of NDV strains, only minor antigenic differences can be detected with standard serological tests (Marin *et al.*, 1996). Virulence is the main character of NDV that has attracted the attention of researchers in their studies. Among the paramyxoviruses, NDV is unique in that the significant variation in virulence within the same serotype has been described (Waterson *et al.*, 1967).

The role of conventional tests in the determination of virulence of ND viruses is still significant but with some limitations. A source of eggs and chickens, which should preferably be from a specific-pathogen-free flock, is needed. Confirmed diagnosis may be slow, taking several days to isolate the virus and carry out the pathogenicity tests. In addition, the use of animals in this way is becoming increasingly unacceptable with the development of actual or potential alternatives (Aldous and Alexander, 2001). As a result from the progress made in molecular biological techniques especially after the invention of polymerase chain reaction (PCR) by Kary Mullis (1985), attempts have been focused on genetic studies of viral pathogens as well as ND