RANDOM MUTAGENESIS OF NS1 PROTEIN OF INFLUENZA A H1N1 AND DOCKING OF RNA APTAMERS TO WILD TYPE AND MUTANT NS1 PROTEINS

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To my dearest family and friends,

who gave me inspiration and endless support

all along.

Thank you.

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ABSTRACT

The NS1A protein is a non-structural protein from influenza A virus H1N1 strain. The protein is a multifunctional protein which is capable of blocking the defense mechanism of host immune by inhibiting the secretion of host cell IFN α/β. Even existing vaccines cannot protect host cells against this viral infection due to constant mutations of NS1A protein. In this study, the NS1A gene which was formerly cloned in pET 32c(+) vector was successfully mutated using error-prone PCR with increased concentration of $MgCl_2$ to 10 mM and subsequently cloned into yT&A vector and transformed into *E. coli* DH5α. There were four proteins that contain non-conservative mutations from sequencing which were NS1 F103LN209D, NS1 S7P, NS1 T76I and NS1 E159G mutant proteins. These proteins together with the wild-type protein were modeled using EasyModeller 2.1 and were energy minimized using GROMACS. The qualities of the structures were validated using ERRAT, PROCHECK, Verify3D and ProSA web. All the structures were of good quality and the high RMSD value shows that the mutant proteins have low structural homology to the wild-type protein. This proves that the structures were affected by point mutations. None of the mutations fell into 'hot spot' mutations. These proteins were subsequently docked to RNA aptamers via HEX server to analyze the binding regions and binding affinity of aptamers to proteins. The results obtained shows that the protein mutations affect the binding properties of aptamers to the mutant proteins because aptamers were docked at various regions with different binding affinities. The aptamers with the highest binding affinity towards wild-type NS1A protein and mutant proteins were selected which were aptamers 21, 174 and 176. These results were expected to be useful for potential drug design to curb future H1N1 viral infections.

ABSTRAK

Protein NS1A merupakan protein nonstruktural dari virus influenza A H1N1. Protein ini ialah protein multifungsi yang boleh menghalang mekanisma pertahanan sel hos dengan menyekat penghasilan IFN α/β. Vaksin yang sedia ada tidak boleh melindungi sel-sel terhadap jangkitan virus sebab protein NS1A ini sentiasa melalui mutasi berterusan. Dalam kajian ini, gen NS1A yang diklon dalam vektor PET 32c (+), telah berjaya dimutasikan menggunakan *error-prone PCR* dengan meningkatkan kepekatan $MgCl_2$ kepada 10 mM dan seterusnya diklonkan ke dalam vektor y T&A dan ditransformasikan ke dalam *E. coli* DH5α. Terdapat empat protein yang mengandungi mutasi bukan-konservatif dari analisa *sequencing* iaitu NS1 F103LN209D, S7P NS1, NS1 T76I dan NS1 E159G protein mutan. Protein ini bersama dengan protein *wild-type* telah dimodelkan menggunakan EasyModeller 2.1 dan tenaga telah dikurangkan menggunakan GROMACS. Struktur kualiti protein-protein telah disahkan dengan menggunakan ERRAT, PROCHECK, Verify3D dan Prosa web. Semua struktur protein adalah berkualiti tinggi dan nilai RMSD yang tinggi menunjukkan bahawa protein-protein mutan mempunyai struktur homologi yang rendah terhadap protein *wild-type*. Ini membuktikan bahawa struktur protein dipengaruhi oleh *point mutation*. Tiada mutasi dikenalpasti sebagai mutasi *'hot spot'*. Seterusnya, *docking* antara protein dan aptamer-aptamer RNA dilakukan melalui HEX *server* untuk menganalisis kawasan *docking* dan afiniti *dock* aptamer-aptamer kepada protein. Keputusan menunjukkan bahawa mutasi protein mempengaruhi docking antara aptamer-aptamer dan protein-protein mutan kerana aptamer-aptamer telah *dock* di pelbagai kawasan dengan kekuatan *docking* berbeza. Aptamer-aptamer yang *dock* kepada protein *wild-type* NS1A dan protein-protein mutan dengan afiniti paling tinggi telah dipilih iaitu aptamer 21, 174 dan 176. Keputusan ini dijangka berguna bagi rekabentuk ubat yang berpotensi untuk mencegah jangkitan virus H1N1 masa depan.

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LIST OF SYMBOLS/ ABBREVIATIONS/ NOTATIONS/ TERMINALOGY

A	-	Adenine
Amp^r	-	Ampicillin resistant
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pairs
C	-	Cytosine
CASP	-	Critical Assessment of Structure Prediction
CPSF30	-	30-kDa subunit of the cellular cleavage and polyadenylation specificity factor
dH_2O	-	Distilled water
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxynucleotide triphosphates
dsRNA	-	Double stranded RNA
E. coli	-	*Escherichia coli*
ED	-	Effector domain
eIF4F	-	Translation initiation factor
ELISA	-	Enzyme-linked immunosorbent assay
EP-PCR	-	Error-prone polymerase chain reaction
EtBr	-	Ethidium bromide
g	-	Gram
G	-	Gravitational force
G	-	Guanine
G-factor	-	Goodness factor
GUI	-	Graphical User Interface
h	-	Hour
HA	-	Hemagglutinin
H	-	Histidine

IFN	-	Interferon
IPTG	-	Isopropyl-β-D-thiogalactoside
K	-	Kelvin
K_d	-	Dissociation constant
kDa	-	Kilo Dalton
kJ	-	Kilo Joule
L	-	Liter
LB	-	Luria-Bertani
m	-	Mille
MFE	-	Minimum free energy
ml	-	Milliliter
mg/ml	-	Milligram/milliliter
Mg^{2+}	-	Magnesium ion
Mn^{2+}	-	Manganese ion
$MgCl_2$	-	Magnesium chloride
$MnCl_2$	-	Manganese chloride
mmol/L; mM	-	Milli molar
mRNA	-	Messenger RNA
NA	-	Neuraminidase
NaCl	-	Sodium chloride
NCBI	-	National Center for Biotechnology Information
NEP	-	Nuclear export protein
NES	-	Nuclear export signal
NLS	-	Nuclear localization sequence/signal
NoLS	-	Nucleolar localization signal
NMR	-	Nuclear magnetic resonance
ns	-	Nano second
No.	-	Number
NS1	-	Nonstructural protein 1
OAS	-	Oligo (A) synthetase
PABP	-	Poly (A)-binding protein
PCR	-	Polymerase chain reaction
PDB	-	Protein Data Bank
PI3K	-	Phosphatidylinositol 3-kinase

PKR	-	Protein kinase R
ProSA	-	Protein Structure Analysis
ps	-	Pico second
RBD	-	dsRNA-binding domain
RMSD	-	Root mean square deviation
RNA	-	Ribonucleic acids
RNP	-	Ribonucleoprotein
rpm	-	Rounds per minute
s	-	Seconds
SDS-PAGE	-	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SELEX	-	Systematic evolution of ligands by exponential enrichment
ssRNA	-	Single stranded RNA
T	-	Thymine
TAE	-	Tris-Acetate electrophoresis buffer
Taq	-	*Thermus aquaticus*
Trp	-	Tryptophan
µl	-	Microliter
µg/ml	-	Microgram/milliliter
µM	-	Micro molar
U	-	Uracil
UV	-	Ultraviolet
v	-	Volt
vRNP	-	Viral ribonucleoprotein
WHO	-	World Health Organization
wt	-	Wild-type
w/v	-	Weight/volume
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
3D	-	Three-dimensional
Å	-	Angstrom
A	-	Alpha
B	-	Beta
°C	-	Degree Celsius
γ	-	Gamma
δ	-	Delta

ε	-	Epsilon
ζ	-	Zeta
η	-	Eta
Φ	-	Phi
Ψ	-	Psi

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CHAPTER 1

INTRODUCTION

1.1 Background Study

The influenza A H1N1 virus originally from swine, is capable of infecting humans. It is a zoonotic virus, where it can be transmitted from animals to humans and it is classified within the family *Orthomyxoviridae* (Hale *et al*., 2008). However, human-to-human transmission is possible when the influenza A H1N1/2009 virus emerged (Michaelis *et al*., 2009). The disease is so widespread due to high capability of being transmitted via airborne particles. This is the reason why this seasonal influenza gained much attention worldwide in 2009. According to World Health Organization (WHO), the influenza A H1N1/09 virus initially originated from Mexico on the 18th of March, 2009. Since then, this contagious disease had been spreading across oceans and many countries were affected until it had been officially declared as pandemic. As of the 17th October 2009, it was reported that there were more than 414, 000 confirmed cases and nearly 5000 have died due to the disease outbreak (WHO, 2009).

The first disease ever occurred caused by influenza A H1N1 virus was the Spanish flu which occurred in 1918, where it caused the death of more than 40 million people (Reid & Taubenberger, 2003). Another two serious outbreaks occurred after the Spanish flu was the Asian flu which occurred in 1957 and the Hong Kong flu in 1968 (reviewed by Khanna *et al*., 2009). The most recent 2009 outbreak was caused by novel influenza A H1N1 strain that have been genetically

evolved. The triple reassortment of the viral genes came from human, swine and avian host source. (Khanna *et al*., 2009).

The influenza A H1N1 virus contains 8 segments of negative sense single-stranded RNA which code for 12 proteins notably nucleoprotein (NP), nonstructural protein 1 (NS1), nuclear export protein (NEP), matrix protein 1 (M1), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2), PB1-F2, PB1 N40, ion channel protein (M2), haemagglutinin (HA) and neuraminidase (NA) (Potter, 2002).

1.2 Problem Statement

Inefficient proofreading ability of H1N1 viral polymerases leads to increased frequency of mutations that establishes diverse strains (Reid and Taubenberger, 2003). Due to the genetic mutations leading to 'antigenic drifts' (Potter, 2002) and occasional 'antigenic shifts' (reviewed by Rappuoli and Giudice, 2011), the virus becomes highly pathogenic in nature in which the population has little or no immunity to fight against viral infections. Even vaccines produced will no longer be effective in preventing infections caused by "newer" H1N1 strains because conformational changes of the virus obscures antibody binding (Rappuoli and Giudice, 2011; Ghedin *et al*., 2005). So, the disease outbreak will most likely become pandemic if a large human population gets infected and contracted with serious respiratory problems.

The NS1A protein in particular, is a multifunctional protein that contributes to the pathogenicity of the H1N1 virus. NS1A protein increases viral replication upon infection into the host cell and inhibits the production of host interferon (IFN) type I response (Richt and Garcia-Sastre, 2009; Hale *et al*., 2008). This study attempted to generate and investigate the potential of mutant NS1A proteins which were to be used for ligand selection. The NS1 gene was randomly mutated in order to predict future mutations of the protein which could possibly be significant in

preparation of future outbreak. Apart from that, the structures of NS1 proteins successfully generated through random mutations were predicted and used for *in silico* screening against pre-selected RNA aptamers via molecular docking. Aptamers that bind to both wt NS1A protein and mutant NS1A proteins at correct conformations can be analyzed and selected.

1.3 Research Objectives

The objectives of this study were:

1) To mutate the influenza A H1N1 NS1 gene using error-prone PCR with varying concentrations of $MgCl_2$, $MnCl_2$ and increasing number of PCR cycles.
2) To analyze the mutated sequence of NS1A genes using bioinformatics tools.
3) To predict the tertiary structures of the mutant NS1A proteins and RNA aptamers using bioinformatic tools.
4) To select high affinity RNA aptamers via *in silico* docking to wild-type and mutant NS1A proteins.

1.4 Research Scope

There were several parts of research activity in this project including mutagenesis, cloning, multiple sequence alignment, protein modeling and molecular docking. The NS1A gene from clone 104 of pET-32c(+) vector in *E.coli* BL21(DE3) strain were mutated using error-prone PCR. The mutated amplicons were further cloned in yT&A cloning vector and transformed into *E. coli* DH5α. In this project, the mutants were analyzed using various bioinformatics tools and this included protein modeling and molecular docking of mutant proteins to RNA aptamers to examine whether the structures of mutants affect docking properties as well as to select RNA aptamer for high affinity binding to NS1A protein.

1.5 Research Significance

The benefit from the outcome of this study is that RNA aptamers with high binding affinity to wt NS1A protein as well as mutants can be chosen as the molecular diagnostic tool or antiviral agent against H1N1 infections. The aptamers with high binding affinity to the specific viral proteins can be used as an alternative to the stable vaccines and antibodies since the pathogenic influenza A H1N1 virus is constantly evolving to circumvent host immunity. As the existing vaccines may no longer be effective in preventing future H1N1 outbreak, novel RNA aptamers obtained from this study may prove to be useful ligand in the future.

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