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Original Article

Vaccine development against the matrix gene (M-Protein) of influenza virus type-A, subtype-H5N1

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Diagnosis of Influenza A Virus Subtype H5N1 by Immunological Tests.
A-Agglutination test
b)Precipitation test.
Isolation of DNA from Blood Samples of Homo sapiens (Human) and Gallus gallus (Chicken).
Amplification & Sequencing of the Matrix gene (M2protein).
Vaccine designing using M2 protein with the Reverse vaccinology approach in Bioinformatics.

ABSTRACT

Influenza is one of the prominent diseases of interest to the research domain because of its constant changes in the antigenic property of the virus. Therefore there is a need to develop strain specific influenza vaccines. Current work is on the study of pathogenicity of H5N1 strain in causing Avian Influenza (AI) and the vaccine development against the disease using the reverse vaccinology approach. To study the pathogenicity of the disease various assays were performed using Immuno techniques like Agglutination& Precipitation tests. The Matrix Protein of Influenza virus is of high consideration and has been reported to play a prominent role in creating pathogenicity. The matrix2 protein was taken into consideration for our study because this protein was found to be conserved in many influenza strains. DNA Isolation was done from the avian influenza infected blood samples of human and chicken. The matrix2 (M2) gene sequence is amplified using (PCR) Technique and sequenced. The work was then extended towards development of subunit vaccine (peptide vaccine) using the Reverse vaccinology approach of bioinformatics. The antigenicity studies, epitopic prediction and surface accessibility area calculation are steps required for deciding the best antigenic peptides. All the above analysis is performed for the matrix2 protein of human and chicken. The antigenic peptides were designed and secondary structure prediction was done. Based on the energy of the structure we can predict the best antigenic peptide which has the capability of being a potent peptide vaccine against Influenza A virus subtype H5N1 which cause a zoonotic disease called Avian Influenza (AI).

1. Introduction

Viruses are infectious agents of small size and simple composition that can multiply only in living cells of animals, plants, or bacteria. "The earliest indications of the biological nature of viruses came from studies in 1892 by the Russian scientist Dmitry I. Ivanovsky and in 1898 by the Dutch scientist Martinus W. Beijerinck. (Kara et al., 2011).

Viruses are classified by the type of nucleic acid they contain, chemical and physical properties, shape, structure, host range, and how they replicate.

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DNA viruses:

Are viruses that have DNA but no RNA. Common DNA viruses are: Hepadnaviruses, Herpesviridae, Poxviridae, Adenoviridae

RNA viruses: Are viruses that contain RNA but not DNA. Common RNA viruses are: Flaviviridae , Retroviridae, Picornaviridae, Orthomyxoviruses, Rhabdovirus.

Oncogenic viruses: Are viruses that produce tumors when they infect humans. The more common oncogenic viruses are: Human papillomavirus (HPV), Herpes simplex virus 2(HSV2), Epstein - Bar Virus (EBU)(Tom Betsy et al., 2005).

Despite the abundance of exotic names given to influenza viruses and their somewhat mystical and confusing nomenclature, there are in fact only three types of influenza virus: type A, B and C, the former of which can be divided into a range of subtypes. They

all belong to the Orthomyxoviridae family of viruses. (Jonathan Van-Tam et al., 2010).

The influenza A, B and C viruses belong to the genus Influenza virus in the family Orthomyxoviridae, within the negative-sense RNA virus order Mononegavirales.

Influenza viruses grow on embryonated eggs or mammalian cell culture, and when examined in the electron microscope they are seen as approximately spherical particles with a diameter of 80–120 nm. After serial passage in the laboratory, some strains produce filamentous particles, and pleomorphic forms are not uncommon(Stuart-Harris et al., 1985).

Each virus particle is composed of approximately 1% RNA, 70–75% protein, 20–24% lipid and 5–8% carbohydrate. (Arie J. Zuckerman et al., 2009).

Avian influenza (AI) is an infectious viral disease of birds (especially wild water fowl such as ducks and geese), often causing no apparent signs of illness. AI viruses can sometimes spread to domestic poultry and cause large-scale outbreaks of serious disease. Some of these AI viruses have also been reported to cross the species barrier and cause disease or subclinical infections in humans and other mammals. AI viruses are divided into two groups based on their ability to cause disease in poultry: high pathogenicity or low pathogenicity. (WHO 2011).

The H5N1 virus subtype - highly pathogenic AI virus- first infected humans in 1997 during a poultry outbreak in Hong Kong SAR, China. Since its widespread re-emergence in 2003 and 2004, this avian virus has spread from Asia to Europe and Africa and has become entrenched in poultry in some countries, resulting in millions of poultry infections, several hundred human cases, and many human deaths. (Geo F et al., 2010).

When the virus particle is taken up in the endosome, the activity of the M2 ion channel is increased so that ions flood into the particle, inducing a low ph. As a result of this, the HA-M1 linkage is disturbed, the particle opens, the fusion peptide within the HA is Trans located, and the HA fuses with the inner layer of the endosome membrane. The ribonucleoproteins are liberated into the cytoplasm of the cell and transported to the nucleus, where the complex is disrupted, and viral RNA synthesis is initiated. (Bernd Sebastian Kamps et al., 2006).

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism, and is often made from weakened or killed forms of the microbe, (Plotkin S et al., 2008).

Types of vaccines

Live attenuated Vaccines, Inactivated Vaccines, Subunit Vaccines, Toxoid Vaccines, Conjugate Vaccines, DNA vaccines. (NIAID 2012).

Reverse vaccinology

Conventional approaches to develop vaccines are based on the cultivation of the microorganisms in vitro and only abundant components can be isolated by using biochemical and microbiological methods. Although successful in many cases, these approaches have failed to provide vaccines against pathogens that did not have obvious immunodominant protective antigens. With the advent of whole-genome sequencing and advances in bioinformatics, the vaccinology field has radically changed, providing the opportunity for developing novel and improved vaccines.

A new approach to identify vaccine candidates was proposed on the basis of the genomic information. This approach was called reverse vaccinology. (Manmohan Singh et al., 2011).

2. Materials and methods:

In this study I have taken (24) blood samples, 12 samples from human[6 infected and 6 non infected] and 12 samples from chicken[6 infected and 6 non infected].

2.1. Diagnosis of Influenza A Virus Subtype H5N1 by Immunological Tests:

The infected and normal samples has been classified by following two qualitative methods:

a) Agglutination test.

b) Precipitation test.

2.2. Isolation of DNA from blood Samples:

Blood DNA isolation from both infected and Non-Infected samples has been done by Sambrook's methods and Bunce method.

2.3. Agarose Gel Electrophoresis:

To determine the presence of DNA isolated from blood samples and to ensure that a contamination free extraction has been achieved.

2.4. Primer designing:

To design primer for specific gene we use tools like (primer-3 tool) and Invitrogen primer design tool.

2.5. Amplification and Sequencing:

Amplification of the M2 gene has been performed on ABI 9700 PCR system.

2.6. Gene sequences:

After purification PCR product were sequenced under ABI 3500 sequencer,

2.7. Tools and Databases:

BLASTp, TFASTy, ANTIGENIC EMBOSS, Protein variability server, Surface accessibility area, SOPMA, ArgusLab, ORF finder (open reading frame).

3. Results And Discussion

Results of wet lab.

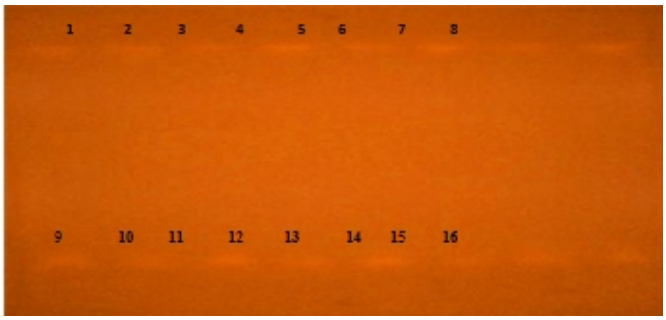

Fig (3.1) G-DNA OF BLOOD SAMPLES FROM Infectious & non-infectious

First 6 samples from infected human and (7,8) control human samples

(9-14) samples from infected animal and (15,16) control animal samples

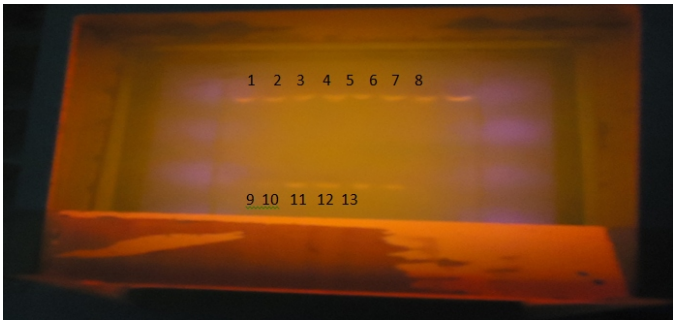

Fig (3.2)PCR GEL PICTURE/ first 6 samples are from infected human and from (7-14) from infected animal.

Bioinformatics Analysis Results

After the M2 Gene amplification and Sequencing, the M2 gene sequences of human and chicken were used for the continuation of the work through Bioinformatics approach.

In Bioinformatics using reverse vaccinology approach the peptide vaccine (subunit vaccine) designing is done. The Source for developing the vaccine is the M2 protein of human and chicken. Thus the gene sequences available through sequences were translated to protein sequences using the Translation tool in Bioinformatics(Gene to Protein Translation using

ORF FINDER tool).

Table(3.1)There are 3 antigenic determinants in M2 protein sequence(human):

Start Position	Sequence	End Position
15	WECRCSD	21
23	SDPIVVAANIIGILHLILWILDRLFFKCIYRRLKY	57
80	QQSAVDVDD	88

Inference: From Epitope prediction, the peptides WECRCSD ,SDPIVVAANIIGILHLILWILDRLFFKCIYRRLKY and QQSAVDVDD was found toBe the .epitopic regions of the M2 Protein of Human.

Table (3.2)There are 2 antigenic determinants in M2 protein sequence(Chicken):

Start Position	Sequence	End Position
16	WECRCSDSSDPLVVAASIIGILHLILWILDRLFFKCIYRRLKY	58
83	NAVDVDD	89

Inference: From Epitope prediction, the peptidesWECRCSDSSDPLVVAASIIGILHLILWILDRLFFKCIYRRLKY and NAVDVDD was found to be the epitopic regions of the M2 Protein of Chicken.

Table (3.3) List of peptides along with their energies

PEPTIDE	Source	Energy
WECRCSD	Human	89.18kcal/mol
SDPIVVAANIIGILHLILWILDRLFFKCIYRRLKY	Human	543.14kcal/mol
QQSAVDVDD	Human	69.25kcal/mol
WECRCSDSSDPLVVAASIIGILHLILWILDRLFFK CIYRRLKY	Chicken	621.50kcal/mol
NAVDVDD	Chicken	50.35Kcal/mol

4. Conclusion

The current study lays the emphasis on the pathogenic /infectious nature of H5N1 in the blood samples of Homo sapiens (Human) and Gallus gallus (Chicken). The pathogenecity was determined by Immuno techniques like Agglutination & Precipitation tests, the matrix gene (M2 gene) was amplified from the Infected and non-Infected (human and chicken) blood samples successfully. The work was further extended to check the antigenicity of the M2 protein and designing a peptide vaccine (subunit vaccine) against H5N1 using one of the Bioinformatics approach named Reverse vaccinology. The M2 protein of H5N1 was analyzed for their antigenicity against Human and Chicken, the most epitopic regions were determined. 5 epitopic regions were analyzed (3 from Human and 2 From Chicken). The Surface accessibility area of the peptides was calculated. Secondary structures of the peptides were predicted. The peptides were

designed and the energy of the structure was analyzed. The peptide QQSAVDVDD (of Human), NAVDVDD (of Chicken) were found to be the potent vaccine candidates and can be used against the attack of H5N1 to Humans and Chicken respectively.This work can be further extended towards Clinical trials which supports the use of this sub unit vaccine in the vaccine development division of the therapeutics industries.

5. Acknowledgement

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