Cloning and Expression of Nonstructural Gene 1 (NS1) of Local Egyptian Strain H5N1

A Thesis Submitted
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Abstract

The highly pathogenic avian influenza (HPAI-H5N1) is a viral contagious disease that affects poultry industry and human health. The rapid and accurate detection of avian influenza virus is a necessary tool for control of outbreaks and surveillance, the vaccination has been considered as a preventive tool in the eradication of AI, but it causes some limitations including trade embargoes and interfering with serologic surveillance in differentiation between infected and vaccinated animals (DIVA strategy).

In this study the open reading frame of NS1 gene of the Egyptian virus A/chicken/Egypt/01112-NLQP/2011 (H5N1) was used as a template to produce DNA clone of the NS1 gene via reverse transcriptase synthesis of cDNA by PCR amplification of the NS1 gene. PCR Products were cloned into pJET1.2® plasmid and subsequently sub cloned into pQE2® expression vector to construct a recombinant plasmid. The Recombinant plasmid designated as pQE2®-NS1 gene was confirmed by PCR colony screening, restriction enzyme digestion, and nucleotide sequence analysis. The recombinant plasmid was transformed into DH5α E. coli strain by heat shock and expressed protein was isolated either a native or denatured form and further purified by immobilization on a nickel ion affinity column. Then the fusion protein was identified by SDS-PAGE, western Immunoblotting revealed the antigenic band of 26kDa NS1 of HPAI using polyclonal antibody. From this work we established a rapid, easy, and reproducible protocol for cloning and expression of (NS1) gene of H5N1 Egyptian virus, and indicated that the NS1 protein suitable in a DIVA for differentiation of AI infected and vaccinated chickens and further applications.

Key words: highly pathogenic avian influenza, nonstructural protein, NS1 expression, NS1 purification.
Dedication

Dedicated to my

Husband

.....My son

.....And my family
Acknowledgment

First and always, all thanks to Allah, Almighty, the most merciful and the compassionate. His guidance and sustenance made this study a reality and came to the light.

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# List of content

## Content

- **Introduction**
- **Review of literature**
  - Historical background
  - History of AI worldwide
  - History of AI in Egypt
  - Evolution of the virus in Egypt
  - Human health significance
  - Causative agent
  - Classification and nomenclature
  - Strain nomenclature
  - Physicochemical proprieties
  - Morphology and structure of virus
  - Genomic organization
  - Virus stability
  - Functional mapping of individual Influenza A virus genes.
  - Polymerase subunits PB2, PB1, PA
    - PB1 Polymerase.
    - PB2 Polymerase.
    - Hemagglutinin
    - Nucleoprotein
    - Neuraminidase
    - Matrix
    - Nonstructural gene
    - NS1 protein
    - Synthesis and biochemistry of NS1
    - Structure of NS1 protein
      - N-terminal ‘RNA-binding’ domain
      - Structure of the C-terminus
    - Functions of NS1 protien
      - NS1 and the host innate immune response
      - Inhibition of Interferon Synthesis
        - Pre-transcriptional limitation of IFN-b induction by NS1
• Post-transcriptional limitation of IFN-b induction by NS1
  ➢ NS1 inhibits intracellular sensors RIG-I
  ➢ NS1 inhibits host mRNA processing and export
  ➢ Selective translation of viral mRNAs
  ➢ NS1 and the host RNA pathway
  ➢ NS1 limits the activity of dsRNA-dependent serine/Threonine protein kinase R (PKR) and 5-oligoadenylate synthetase (OAS)
  ➢ NS1 and the heterodimeric lipid kinase (PI3K) signaling pathway
  ➢ NS1 and the host apoptotic response
  • NS1 and the host adaptive immune response
  • Cell signaling and virulence
  • Intracellular localization of NS1
• Invetro Synthesis and biochemistry of NS1
  • Prokaryotic system
  • Eukaryotic systems
    ➢ Yeast systems (Saccharomyces cerevisiae, PichiaPastoris)
    ➢ The baculoviral expression system
  • Mammalian cell expression system
  • Vectors used in NS1 protein expression
  • Virus replication
  • Pathogenesis and Pathogenicity
  • Antigenic variation of AI virus strain
  • The immune response to influenza infection
  • Diagnosis of avian influenza
  • Clinical signs
  • Postmortem lesions
  • Laboratory diagnosis of influenza H5N1
    ➢ Isolation in embryonated chicken eggs-(ECE)
    ➢ Isolation in cell culture
  • Molecular diagnosis
    ➢ Conventional PCR/reverse transcriptase-PCR
    ➢ Real-time PCR/RT-PCR
    ➢ Loop-mediated isothermal amplification
    ➢ DNA microarray
    ➢ Nucleic acid sequencing-based amplification method
    ➢ Sequence Analysis
    ➢ Pyrosequencing
- Serological tests for AI antibody detection
- Haemagglutination & Haemagglutination Inhibition test
- Neuraminidase inhibition (NAI) assay
- Agar gel immunodiffusion
- Enzyme Linked Immunoassay
  - Enzyme immunoassays and use a monoclonal antibody against the nucleoprotein.
  - Enzyme immunoassays and use a monoclonal antibody against the nonstructural protein 1
- Strain virulence (pathogenicity test)
- Detection and identification of the viral protein
  - Sodium dodecyl sulfate polyacrylamide gel electro-phoresis (SDS-PAGE)
  - The western blot
- Avian Influenza Control Strategies
  - Vaccination
  - Conventional inactivated vaccine
  - Recombinant vaccine
  - Reverse genetics inactivated vaccine
  - non-structural (NS) genes as a live influenza vaccine
  - Replication defective vaccine (VLP)
  - DNA vaccine
  - Universal Vaccines
  - Antiviral drugs
    - Antiviral compounds targeting functions of the NS1 protein
Material and Methods
- Material
  - Avian influenza virus
  - Material used for avian influenza virus isolation
  - Material required for Hemagglutination (HA) test
  - Material and equipments used for Extraction of the RNA from isolate
  - Material used for Real –Time PCR (RT-PCR)
  - Material used for Polymerase Chain Reaction (two step RT-PCR)
  - Material used for Agarose Gel Electrophoresis
  - Equipment used in two step Polymerase Chain Reaction- RT-PCR
  - Materials used for PCR product Purification
  - Materials used for sequencing of NS1 gene
Material used for cloning of NS1 gene
- PCR cloning kit
- Bacteria used for transformation of NS1 gene
- Restriction enzyme
- Shrimp Alkaline Phosphatase (SAP)
- Expression vector
- Media for bacterial cultures
- Antibiotics use for selection
- Equipment use for cloning
- Material used for plasmid extraction
- Material used for Inducing NS1 protein Expression
- Material used for purification and detection of recombinant tagged protein
- Material used for detection of recombinant tagged protein using SDS-PAGE
- Molecular weight determination by SDS-PAGE
- Material used for Coomassie Staining SDS-PAGE
- Materials and equipment used for Immunodetection of tagged NS1 Proteins
- Materials and equipment used for Agar gel immunodiffusion.
- Materials and equipment used for Western blottin.

Methods
- Hemagglutination test (HA)
- Methods of Extraction of the RNA
- Procedure of Real Time-PCR (RRT-PCR)
- Method of NS1 genes amplification
- Methods of Reverse Transcriptase
- Method of Polymerase Chain Reaction (PCR):
- Methods for purification of the PCR Products
- Method of sequencing the NS1 gene
- Method of cloning of NS1 gene
- Method of Transformation of DH5α E.coli with cloned NS1 gene
- Method of screening recombinant plasmid with NS1
- Method for plasmid extraction
- Method of Digestion by SALI restriction enzyme
- Method of Shrimp Alkaline Phosphatase (SAP)
- Method of Sticky end Ligation
- Method for Transformation of DH5α E.coli with recombinant PQE2®
- Method for confirming proper orientation of NS1 in PQE2.
- Method of Plasmid extraction for PQE2®
• Method of Sequencing the insert in extracted recombinant PQE2 plasmid: 82
• Method of Induction of recombinant NS1 protein Expression 84
• Method of expressed 6xHis-tagged recombinant NS1 Proteins Purification. 85
• Method of recombinant NS1 Proteins Purification Under Native Condition 85
• Method of recombinant NS1 Proteins Purification Under Denaturing 86
• Method of Separation of Proteins by SDS-PAGE 87
• Method of Coomassie Staining of SDS-PAGE 88
• Method for Immunodetection of 6xHis-tagged 89
  • Agar gel immunodiffusion. 89
  • Method of Western Blotting 89

❖ Results
• Result of virus propagation 91
• Results of RT-real time PCR 92
• Amplification curve for AIV H5 gene RRT-PCR 92
• Results of RT-PCR for NS1 gene 92
• Result of Transformation of cloned NS1 gene 93
  • Analyzing Transformants (screening recombinant plasmid with NS1) 93
  • Result of p-jet 1.2 plasmid extraction 94
• Result Digestion by SALI restriction enzyme 95
  • Result OF Recombinant pJET1.2 Cloning Vector Digestion by SALI 95
  • Result OF Digestion of PQE2 Expression Vector by SALI enzyme 96
• Result of Transformation of recombenent PQE2-NS1 in DH5αE.coli 96
• Result of confirming proper orientation of NS1 in PQE2 97
  • Colony-touch PCR 97
  • Confirmation by sequencing of recombinant PQE2with NS1 gene 98
• Induction of expression with isopropyl-β-D thiogalactoside (IPTG)) 101
• Result of Spectrophotometric measurement of expressed 6xHis-tagged NS1 101
• Result of Separation of 6xHis-tagged NS1 Proteins by SDS-PAGE. 102
  • Under Native Conditions 102
  • Under Denaturing Conditions 102
• Result of Immunodetection of tagged NS1 Proteins by using Western Blotting 102
• Result of tagged Proteins by using Agar gel Immunodiffusion. 102

❖ Discussion 104
❖ English summary 115
❖ References 117
❖ Arabic summary 1
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Influenza A virus genome RNA segments and coding assignments</td>
</tr>
<tr>
<td>2</td>
<td>Available drugs against H5N1 virus and their modes of action</td>
</tr>
<tr>
<td>3</td>
<td>Sequence of H5 primers and probe used in Real-Time PCR</td>
</tr>
<tr>
<td>4</td>
<td>Real-Time RT-PCR the H5 Subtype</td>
</tr>
<tr>
<td>5</td>
<td>Thermo cycling Conditions for H5.</td>
</tr>
<tr>
<td>6</td>
<td>Reaction mix of Reverse Transcriptase-Polymerase</td>
</tr>
<tr>
<td>7</td>
<td>Thermal profile used in Reverse Transcriptase-step</td>
</tr>
<tr>
<td>8</td>
<td>Primers used (PCR) amplification of NS1 gene</td>
</tr>
<tr>
<td>9</td>
<td>Reaction mix of Polymerase Chain Reaction by Phusion hot start</td>
</tr>
<tr>
<td>10</td>
<td>Thermal profile used in Polymerase Chain Reaction by Phusion hot start</td>
</tr>
<tr>
<td>11</td>
<td>Ligation mix of NS1 gene and pJET1.2/blunt Cloning Vector</td>
</tr>
<tr>
<td>12</td>
<td>Reaction mix of screening for NS1 with in P-Jet1.2</td>
</tr>
<tr>
<td>13</td>
<td>Thermal profile used in Polymerase Chain Reaction (PCR) thermo two step</td>
</tr>
<tr>
<td>14</td>
<td>Sequence of primers used in screening of cloned NS1</td>
</tr>
<tr>
<td>15</td>
<td>Digestion mix of SALI restriction enzyme</td>
</tr>
<tr>
<td>16</td>
<td>Ligation mix of PQE2 VECTOR and digested NS1</td>
</tr>
<tr>
<td>17</td>
<td>Mix for the colony touch screening</td>
</tr>
<tr>
<td>18</td>
<td>Thermal profile (PCR) for the colony touch screening</td>
</tr>
<tr>
<td>19</td>
<td>Primers used screening of NS1 gene by Colony-touch PCR:</td>
</tr>
<tr>
<td>20</td>
<td>Sequence of primers used in sequence The r-PQE2 and NS1 inserts</td>
</tr>
<tr>
<td>21</td>
<td>Reaction for Big dye Terminator V3.1 cycle sequencing kit.</td>
</tr>
<tr>
<td>22</td>
<td>Thermal profile used of sequence</td>
</tr>
<tr>
<td>23</td>
<td>Preparation of SDS-PAGE gels</td>
</tr>
</tbody>
</table>

List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Structure of avian influenza virus.</td>
</tr>
<tr>
<td>2</td>
<td>The NS1 and nuclear export protein (NEP) mRNAs of the influenza A virus.</td>
</tr>
<tr>
<td>3</td>
<td>Binding sites of cellular proteins on the domains of the NS1A protein</td>
</tr>
<tr>
<td>4</td>
<td>Topology diagram of the C-terminus monomer of the NS1A protein</td>
</tr>
<tr>
<td>5</td>
<td>Interaction between NS1 and host molecules.</td>
</tr>
<tr>
<td>6</td>
<td>Viral inhibition of RNase L</td>
</tr>
<tr>
<td>7</td>
<td>Virus interference of PKR.</td>
</tr>
<tr>
<td>8</td>
<td>Map of pQE-2 Expression vector</td>
</tr>
<tr>
<td>9</td>
<td>Diagram of the influenza viral life cycle</td>
</tr>
<tr>
<td>10</td>
<td>Available drugs against H5N1 virus and their modes of action</td>
</tr>
<tr>
<td>11</td>
<td>Genetic Map of Pjet1.2/Blunt</td>
</tr>
</tbody>
</table>
12 Genetic Map Of PQE2
13 Expressing & Purifying 6xHis-tagged Recombinant Proteins
14 Amplification curve for AIV H5 gene RRT-PCR
15 Gel electrophoresis of the Results of conventional RT-PCR for NS1 gene
16 Transformation of cloned NS1 gene in P-jet 1.2 vectors
17 Gel electrophoresis of screening Results of NS1 gene in P-JET plasmid.
18 Gel electrophoresis of Result of recombinant P-jet 1.2 plasmid extractions.
19 Result of gel electrophoresis of Recombinant pJET1.2 Cloning Vector Digestion by SALI enzyme
20 Agarose gel electrophoresis showing the Result of of PQE2 Expression Digestion by SALI enzyme.
21 Transformation result of DH5α E. coli. rPQE2 with NS1 in competent
22 Gel electrophoresis of screening of positive colony of NS1 gene in PQE2
23 Sequence alignment for the NS1 of the Egyptian isolates and NS1 -PQE2.
24 Coomassie-stained SDS-PAGE 6xHis-tagged NS1 protein under native conditions
25 Stained SDS-PAGE 6xHis-tagged NS1 protein under denaturing conditions
26 Result of Western blot analysis showing 6xHis-tagged NS1 protein
27 Result of Agar gels Immunodiffusion

LIST OF ABBREVIATIONS

A Alanine
AAF Amnioallantoic fluid
AGID Agar gel immunodiffusion
AI Avian influenza
APMV avian paramyxovirus
BLAST Basic Local Alignment Search Tool
BP Base pair
BSL-2 Biosafety level 2
CAS Chorioallantoic sac
cDNA Complementary of Deoxyribonucleic acid
CEF Chicken embryo fibroblast
CPSF Cleavage and polyadenylation specificity factor
cRNA Cellular Ribonucleic acid.
CT Cycle threshold

VII
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DW</td>
<td>Distilled water</td>
</tr>
<tr>
<td>E</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>ECE</td>
<td>Embryonated chicken eggs</td>
</tr>
<tr>
<td>EID50</td>
<td>Embryo infective dose fifty</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic translation initiation factor 2α</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked Immunosorbent assay</td>
</tr>
<tr>
<td>F</td>
<td>Phenylalanine</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>H</td>
<td>Histidine</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutination</td>
</tr>
<tr>
<td>HACS</td>
<td>HA cleavage site</td>
</tr>
<tr>
<td>HAU</td>
<td>hemagglutinating unit</td>
</tr>
<tr>
<td>HI</td>
<td>hemagglutination inhibition</td>
</tr>
<tr>
<td>HPNAI</td>
<td>High Pathogenicity notifiable avian influenza</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>ID</td>
<td>Immunodiffusion</td>
</tr>
<tr>
<td>IFN-Α/Β</td>
<td>Interferon- α/β</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-beta-D-thiogalactopyranoside.</td>
</tr>
<tr>
<td>IVPI</td>
<td>intravenous pathogenicity index</td>
</tr>
<tr>
<td>K</td>
<td>Lysine</td>
</tr>
<tr>
<td>L</td>
<td>Leucine</td>
</tr>
<tr>
<td>LAMP</td>
<td>Loop-mediated isothermal amplification</td>
</tr>
<tr>
<td>LB</td>
<td>LURIA Bertani</td>
</tr>
<tr>
<td>LLC-MK2</td>
<td>rhesus monkey kidney</td>
</tr>
<tr>
<td>LPAI</td>
<td>Low pathogenic avian influenza</td>
</tr>
<tr>
<td>LPCS</td>
<td>Low pathogenic cleavage site</td>
</tr>
<tr>
<td>M</td>
<td>Matrix</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin Darby canine kidney</td>
</tr>
<tr>
<td>MGB</td>
<td>minor groove binder</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MPAI</td>
<td>Mild pathogenic avian influenza</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N</td>
<td>Asparagine</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>NAMRU-3</td>
<td>Naval Medical Research Unit No. 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>NASBA</td>
<td>Nucleic acid sequencing-based amplification</td>
</tr>
<tr>
<td>NEP</td>
<td>Nuclear export protein</td>
</tr>
<tr>
<td>NLQP</td>
<td>National Laboratory for Quality Control of Poultry Production</td>
</tr>
<tr>
<td>NLSS</td>
<td>nuclear localization signals</td>
</tr>
<tr>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>Nonstructure protein</td>
</tr>
<tr>
<td>OAS</td>
<td>5-oligoadenylate synthetase</td>
</tr>
<tr>
<td>OIE</td>
<td>Office Internationale des Epizooties</td>
</tr>
<tr>
<td>P</td>
<td>Proline</td>
</tr>
<tr>
<td>PA</td>
<td>Polymerase acid</td>
</tr>
<tr>
<td>PAB II</td>
<td>poly(A)-binding protein II</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>PB1</td>
<td>Polymerase basic protein 1</td>
</tr>
<tr>
<td>PB2</td>
<td>Polymerase basic protein 2</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKR</td>
<td>dsRNA-dependent serine/Threonine protein kinase R</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginin</td>
</tr>
<tr>
<td>RBD</td>
<td>RNA-binding domain</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic Acid Inducible Gene I</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid.</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RRT-PCR</td>
<td>Real-Time reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RT-LAMP</td>
<td>Reverse transcriptase-LAMP</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase-PCR</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SEPRL</td>
<td>South east poultry regional lab</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific pathogen free</td>
</tr>
<tr>
<td>ssRNA</td>
<td>Single-Stranded RNA</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris borate EDTA</td>
</tr>
<tr>
<td>TCID50</td>
<td>Tissue culture infective dose fifty</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>vRNA</td>
<td>Viral Ribonucleic acid.</td>
</tr>
</tbody>
</table>
Introduction
1. Introduction

Highly pathogenic avian influenza have significant threat to human public health, due to the risk for human transmission (Schlingemann et al., 2010). The local poultry industry, which had previously produced millions of chickens daily is estimated to have lost of 2–3 billion US$ due to avian influenza. This loss has affected the incomes of the 1·5 million people whose livelihoods depended on poultry (Meliegy, 2007). Furthermore, it was demonstrated that H5N1 subtype could directly cross the species barrier to replicate in humans and cause severe disease (Kodihalli et al. 2000). Recent genetic characterization of H5N1 strains involved in the current panzootic has demonstrated two distinct phylogenetic clades (clades 1 and 2) (WHO, 2008).

AI viruses are influenza A viruses belonging to the Orthomyxovirus family, and they are classified according to their pathogenicity and the antigenicity of the surface proteins haemagglutinin (HA) and neuraminidase (NA) of which 17 and 10 variants, respectively, are known to date (tong et al.,2012). Viruses containing subtypes H5 and H7 are highly pathogenic in poultry and cause outbreaks of highly pathogenic AI (HPAI), with mortality rates reaching 100 % (Webster et al., 1992). The genome of influenza type A virus consist of 8 unique segments of ssRNA which are of negative polarity (Nicholson et al., 2003). These eight different gene segments encode at least 10 different viral proteins. The structural proteins in the mature virion can be divided into the surface proteins that include the HA, NA, and M2 proteins and the internal proteins, including the NP, M1, and the polymerase complex composed of PB1, PB2, and PA, and Two additional proteins produced by influenza viruses are the nonstructural proteins, NS1 and NS2, NS2 is also known as the nuclear export protein (NEP) (Swayne, 2008).

The NS1 protein is a protein of our concern; it is the most important viral regulatory protein that interferes with host native immunity by inhibiting the
interferon-mediated immune response and cellular protein synthesis (Bergmann et al., 2000). It is a multifunctional protein that participates in both protein-protein and protein-RNA interactions. NS1 proteins can be divided into two major groups, originally termed alleles A and B. A number of NS1 proteins from avian influenza viruses together with those of all human, swine and equine influenza viruses are described as allele A NS1 proteins, whereas those of allele B are exclusively from avian viruses (Hale et al., 2008b). The NS1 protein is considered to be a true nonstructural protein that produced in large amounts in the host cell (Swayne, 2008), but is not incorporated into the virion (Wacheck et al., 2010). Thus, the NS1 protein presents only in the infected animals and not in animals vaccinated with the inactivated virus. This makes NS1 a better antigen for use in the development of a diagnostic tool that can differentiate between infected and vaccinated animals (DIVA) (Swayne, 2008).

Direct detection of AI viral proteins or genes can be carried out as well as virus isolation either in embryonated chicken eggs or in tissue culture (Swayne and Halvorson, 2003). This methodology still represents the gold standard and the official method for detection of AI viruses (OIE, 2006). Also the AIV can be identified by RT-PCR and RRT-PCR (Swayne and Halvorson, 2003). RRT-PCR has been described to be 100 fold more sensitive than virus isolation procedures (tong et al., 2012). Viral culture assay is quite sensitive, but time consuming and technically demanding, and requires the presence of infectious viral particles; instead, ELISA for antibodies or antigen is a test of limited specificity (Pachucki, 2007).

Recently, Tumpey et al. (2005) developed an enzyme-linked immunosorbent assay (ELISA) for DIVA on the basis of antibodies to NS1, which could be used as a serodiagnostic tool for the determination of LPAI virus infection of poultry. However, its use was not confirmed for the detection of the HPAI virus infection in
poultry (Tumpey et al., 2005). Furthermore, the recombinant protein-based serological tests may have higher sensitivity and specificity as the target antigen is immune-dominant and devoid of any non-specific moieties present in whole cell preparations (Mohan et al. 2006).

In this study we successfully expressed and purified the 6x His-Tag-Nonstructural protein (NS1) in DH5α E.coli expression system which show to be easy, not complicated system and could be produced in high amount of recombinant protein when compared to other production systems, The protein could be easily purified from a large amount of contaminating soluble bacterial protein these findings strongly recommend that this protein might be useful as a potential antigen for diagnostics against natural/experimental HPAI infections. Because the NS1 is expressed in influenza virus-infected cells, and it is not packaged in the virion, it is an attractive candidate for a DIVA differential diagnostic test (Avellaneda et al. 2010).

**The aims of this study are:**

1. NS1 Gene cloning using P-JET® plasmid.
2. Expression of NS1 protein of AIV HPAI (H5N1) of local Egyptian strain using E. coli expression system and PQE2® expression vector.
3. Characterization of The expressed protein was by SDS-PAGE and conformation by western Immunoblotting.
Review of Literature
2. Review of literature

2- Historical background

2.1-History of AI worldwide

Avian Influenza disease is an infectious disease of birds that is caused by influenza virus type A strains, was identified first in Italy in 1878 (Ligon, 2005). Avian influenza (AI) caused disease of many kinds of poultry, wild and caged birds characterized by marked variation in morbidity, mortality signs and lesions. In addition, the infection causes periodical epidemics in humans, pigs, seals, and a variety of birds (Swayne et al., 2003). The virus also possesses considerable zoonotic potential. Human cases of HPAIV H5N1 infection, characterized by a high fatality rate, started to occur due to virus exposure of humans at the poultry human interface which is highly fissured in Egypt (Fasina et al., 2009).

The first report of H5N2 avian influenza A virus isolated from psittacine bird and represents the first introduction of this virus into the United States (Hawkins et al., 2006). Saudi Arabia, Jordan, Kuwait, Iraq, and Iran (OIE, 2008). Highly pathogenic avian influenza virus A/H5N1 was first officially reported in Africa in early 2006. Since the first outbreak in Nigeria, this virus spread rapidly to other African countries. From its emergence to early 2008, 11 African countries experienced A/H5N1 outbreaks in poultry and human cases were also reported in three of these countries, at present, little is known of the epidemiology and molecular evolution of A/H5N1 viruses in Africa. (Cattoli et al., 2009).

Globally, the number of reported outbreaks of H5N1 in poultry and wild birds increased since mid-2008 between the 2009-2010 and the 2010-2011 time periods, the main increase occurs in Asia, with a significant rise in number of events in some Asian countries and relatively large outbreaks in others (WHO, 2011).
2.2-History of AI in Egypt

Reports of veterinary service in 1912 referred to Fowl plague as an indigenous disease infected the Egyptian poultry. Before that date the disease has been confused with fowl cholera, or typhoid which was prevalent in the country. Rapid spread of fowl plague was recorded in chickens, turkeys, waterfowls, parrots and pheasants (*Rashad, 1934*). *Hosny et al., (1980)* isolated 9 AIV isolates (two was H3N1 and other 7 isolates were H4N1) were obtained and studies of *Amin et al., (1980)*.

From December 2003 through the present, highly pathogenic avian influenza (HPAI) H5N1 virus infections in birds have been reported in Asian, African, European, and Middle Eastern countries (*WHO, 2011*). In February 2006 outbreaks of H5N1 in poultry were confirmed in Egypt, and genetic analyses indicated introduction of a Qinghai Lake-like H5N1 strain (*Saad et al., 2007*). In 2007 the Egyptian Government reported the isolation of Avian Influenza subtype H7 from wild migratory ducks in El-Abassa Lake- El-Sharkia province (*Peiris et al., 2007*). Seven Jul 2008 Egypt reports outbreaks in 9 governorates in commercial and backyard poultry, and poultry in live bird markets. The national veterinary Services (GOVS) declares H5N1 to be endemic in Egypt. In December 2009; Egyptian veterinary authorities reported 21 H5 HPAI outbreaks in poultry (chickens, ducks and turkeys) rates. All of these outbreaks (100%) were reported from the household poultry sector. Fourteen of the 21 outbreaks occurred in non-vaccinated birds, while the vaccination status of the remaining outbreaks remains unknown (*FAO, 2009*).

2.3 Evolution of the virus in Egypt:

Genetic analysis of the Hemagglutinin (HA) gene from 2006 to 2011 was done by sequencing of the full-length H5 gene; the epidemiological pattern of disease outbreaks in Egyptian poultry farms seems to be seasonal with no specific geographic distribution across the country. The molecular data revealed that there are two major groups of viruses: the classic group of subclade 2.2.1