UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A RAPID DIAGNOSTIC TECHNIQUE FOR EQUINE INFLUENZA

ABDUL RAHMAN D. ABDUL HADI

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DOCTOR OF PHILOSOPHY

UNIVERSITI PUTRA MALAYSIA

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By

ABDUL RAHMAN D. ABDUL HADI

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

May 2012

DEDICATION

To the loving memory of my late father Dahham Abdul Hadi, “may Allah bless him with his supreme benevolence” who is forever remembered.

His silent inspiration, encouragement, and guiding hand on my shoulder still linger on.

To my caring, and lovely mother, wife, brother, sisters, and all those who passed away in struggle for sovereignty of my fatherland.

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Abstract of thesis presented to the Senate of University Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

DEVELOPMENT OF A RAPID DIAGNOSTIC TECHNIQUE FOR EQUINE INFLUENZA

By

ABDUL RAHMAN D. ABDUL HADI

May 2012

Chairman: Assoc. Prof. Datuk Bashir Ahmad Fateh Mohamed, PhD

Faculty: Veterinary Medicine

Equine influenza virus (EIV) is a highly contagious and widely distributed respiratory disease of equiadae caused by a type A influenza virus from the family Orthomyxovirus. Influenza in equines is caused by two types of viruses, H3N8 and H7N7. The viruses currently circulating among horses are of the H3N8 sub-type. H7N7 has not been reported for more than three decades from any part of the world. Vaccination against equine influenza, a powerful tool for the control of the disease, may result in issues related to vaccinations interferes with sero-surveillances program of EIV infection.

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The use of vaccination against equine influenza have greatly worldwide acceptance if a reliable test were available that clearly discriminate between naturally infected and vaccinated animals (DIVA). Because horses that were vaccinated with "inactivated vaccines" have not produced non-structural protein (NS1) specific antibodies, while they have presented in the naturally infected horses, therefore, NS1 protein considered as an attractive candidate for a DIVA differential diagnosis test. The objectives of the present study were to detect, identify and develop a diagnostic kit for EIV.

For virus detection, identification and isolation, a total of 162 nasopharyngeal swabs were collected during 2009-2010. Our study showed that the prevalence of viral nucleic acid was detected in 50 out of 162 (31%) nasopharyngeal swabs. All positive samples were subjected for virus isolation in 9-11 days embryonated specific-free-pathogen eggs (SPF) followed by hemagglutination test, and RT-PCR. Embryonic death did not occur during the five passages and all embryos remained alive. However, the results from HA test and RT-PCR were also showed negative results. The failure to isolate the circulating viral antigen was possibly due to horses that were not in the acute phase of the disease during the period when samples were collected, thus they did not shed a live virus, and the samples were collected from a situation of no form of outbreaks.

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With respect to development, of a rapid and reliable diagnostic technique, the NS1 gene of H3N8 subtype was amplified by RT-PCR and expressed in prokaryotic expression plasmid pRSET B in *E. coli* strain BL21 (DE3)plysS after induction with IPTG. The 6x His-tagged recombinant fusion proteins were purified using the Pro Bond™Purification system and the expressed protein was identified by SDS-PAGE and western-blotting. A recombinant protein of approximately 13kDa was produced. The results showed that the recombinant NS1 protein was expressed and the optimal coating concentration was 2.01μg/ml, the optimal serum dilution was 1:100, and the optimal HRP-IgG dilution was 1:10000.

For evaluation and validation of the developed NS1-ELISA, a total of 344 serum samples were collected from two groups of horses, 144 samples from vaccinated and 200 samples from unvaccinated groups. The results of the newly developed NS1-ELISA were compared to Haemagglutination inhibition (HI) test, indirect equine influenza IgG-ELISA (IBL, Germany) and competitive influenza A IgG-ELISA (IDEXX-USA). The results shows the potential superiority of the NS1-ELISA in the differentiation of vaccinated from unvaccinated (infected animals).

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In conclusion, these results demonstrate the potential benefit of a simple, specific ELISA for anti-NS1 antibodies that may have diagnostic value for the equine industries in Malaysia, and also useful method for serological diagnosis to differentiate vaccinated from naturally infected horses. This recombinant NS1-based ELISA could therefore be a good alternative to currently available kits for detection of antibody to EIV.

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

PEMBANGUNAN TEKNIK DIAGNOSTIK PANTAS BAGI MENGESAN INFLUENZA EKUIN

Oleh

ABDUL RAHMAN D. ABDUL HADI

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Pengerusi: Prof. Madya Datuk Bashir Ahmad Fateh Mohamed, PhD

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Virus influenza ekuin (EIV) adalah satu penyakit yang mudah berjangkit dan menyebabkan penyebaran penyakit pernafasan equiadae secara meluas. Penyakit ini disebabkan oleh sejenis virus influenza tip A dari keluarga Orthomyxovirus. Influenza pada ekuin adalah disebabkan oleh dua jenis virus iaitu H3N8 dan H7N7. Virus yang kini tersebarpada kuda adalah daripada subtip H3N8. H7N7 pula telah tidak pernah dilaporkan lebih daripada tiga dekad dari mana-mana negeri di dunia. Pemvaksinan terhadap influenza ekuin merupakan kaedah utama bagi mengawal penyakit tetapi boleh menimbulkan isu berkaitan gangguan pemvaksinan dengan program sero-pengawasan jangkitan EIV.Penggunaan vaksin terhadap influenza ekuin

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mendapat sambutan yang menggalakkan di seluruh dunia sekiranya ujian pengesahan yang sedia ada dapat membezakan dengan jelas antara haiwan yang dijangkiti secara semulajadi dan yang divaksinat (DIVA). Ini adalah kerana, kuda yang telah disuntik vaksin tak aktif tidak menghasilkan protein antibodi bukan struktur khusus (NS1), yang mana ia boleh didapati secara semulajadi pada kuda yang telah dijangkiti. Oleh itu protein NS1 dianggap sebagai pemilihan yang terbaik bagi ujian diagnosis pembezaan DIVA.Oleh itu, objektif utama pada kajian ini adalah untuk mengesan, mengenal pasti dan membangunkan kit diagnostik untuk EIV.

Untuk pengesanan, pengenalpastian dan pengasingan virus, sejumlah 162 sapuan nasofarinktelah dikumpulkan bermula 2009-2010.Kajianmenunjukkan bahawa kelaziman asid nukleik virus telah dikesan pada 50daripada162 sapuan nasofarinks (31%). Semua sampel positif yang didedah untuk pengasingan virus dalam telur berembrio khusus bebas patogen (SPF) 9-11 hari, diikuti dengan ujian hemagglutination, dan RT-PCR. Kematian embrio tidak berlaku dalam tempoh fasa kelima dan semua embrio masih hidup.Walau bagaimanapun, hasil ujian HA dan RT-PCR juga menunjukkan keputusan negatif.Kegagalan untuk mengasingkan antigen virus yang beredar adalah mungkin disebabkan kuda yang tidak berada dalam fasa akut penyakit dalam tempoh semasa sampel dikumpulkan, di mana kuda tidak

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mengeluarkan virus hidup. Sampel mula dikumpul daripada keadaan di mana tiada wabak berlaku.

Dari segi pembangunan teknik diagnosis yang pantas dan berkesan, NS1 gen subtip H3N8 telah ditambahbaik melalui ujian RT-PCR dan dinyatakan dalam ekspresi prokariot plasmid pRSET B dalam bakteria *E. coli*unsur BL21 (DE3) plysS selepas dirangsangkan dengan IPTG. Protein gabungan rekombinan His bertanda 6x telah disuling menggunakan sistem penulenan pro bond™dan protein ekspresi dikenal pasti menggunakan SDS-PAGE dan western blot. 13kDa protein rekombinan telah dihasilkan.

Hasil kajian menunjukkan bahawa protein rekombinan NS1 telah diekspresikan dan kepekatan optima salutan adalah 2.01μg/ml, pencairan darah yang optimum adalah 1:100, pencairan HRP-IgG yang optimum adalah 1:10000 dan masa tindak balas yang optimum untuk antigen dan antibodi adalah 1 jam. Untuk penilaian dan pengesahan NS1-ELISA, yang telah dibangunkan sebanyak 344 sampel serum telah diambil dari dua kumpulan kuda, 144 sampel dari kumpulan yang telah divaksinat dan 200 sampel daripada kumpulanyang tidak divaksinat.Keputusan NS1-ELISA yang baru dibangunkantelah dibandingkan dengan ujian perencatan Haemagglutination (HI), ujian tidak langsung influenza ekuin IgG-ELISA (IBL, Jerman) dan ujian berdaya saing influenza A IgG-ELISA (IDEXX-USA).

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Kajian ini menunjukkan potensi keunggulan NS1-ELISA dalam pembezaan kumpulan divaksinat dan tidak divaksinat (haiwan yang dijangkiti).

Kesimpulannya, keputusan ini menunjukkan potensi bermanfaat yang mudahdan khusus bagi ujian ELISA untuk antibodi anti-NS1 yang mungkin mempunyai nilai diagnostik untuk industri ekuin di Malaysia, dan juga kaedah yang berguna untuk diagnosis serologi untuk membezakan kuda yang dijangkiti EIV dengan kuda yang telah diimunisasi dengan vaksin tidak aktif. Ujian rekombinan NS1 berasaskan ELISA ini boleh menjadi alternatif yang baik untuk kit yang ada sekarang untuk pengesanan antibodi bagi EIV.

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I certify that a Thesis Examination Committee has met on 11 July to conduct the final examination of Abdul Rahman D. Abdul Hadi on his Doctor of Philosophy thesis entitled “Development of a rapid diagnostic technique for equine influenza” in accordance with the Universities and University Colleges Act 1971 and the constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Doctor of Philosophy.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

ABDUL RAHMAN D. ABDUL HADI

Date: 11- July -2012

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LIST OF ABBREVIATIONS

ATCC	American type culture collection
%	Percent
µg	Microgram
µM	Micromolar
Bp	Base pair
BSA	Bovine serum albumin
C-ELISA	Competitive enzyme linked immunosorbent assay
CF	Complement fixation
CMI	Cellular immune responses
CV	Coefficient of variation
ddH_2O	Double-distilled water
DIVA	Differentiate between infected and vaccinated animals
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dsRNA	Double-stranded ribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethlenediaminetetraacetic acid
EI	Equine Influenza
EIA	Enzyme immunoassays
EIV	Equine Influenza Virus

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ELISA	Enzyme linked immunosorbent assay
H	Hour
HA	Haemagglutination
HI	Haemagglutination inhibition
HRPO	Horseradish peroxidase
IBL	Innovation beyond limits
IBV	Infectious bronchitis virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPTG	Isopropyl-B-D-thiogalacosidase
ISCOM	Immune-stimulating complex
Kb	Kilo base
kDa	Kilodalton
LB	Luria-Bertani
MP	Matrix protein
M	Molecular Marker
MCS	Multiple cloning site
MDCK	Madin-Darby Canine kidney
MgCl2	Magnesium chloride
Min	Minutes
Ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid

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MW	Molecular weight
NA	Neuraminidase
NP	Neucleoprotein
Na_2HPO_4	Di-sodium hydrogen phosphate
NaCl	Sodium chloride
NEP	Nuclear export proteins
NP	Nucleoprotein
NS1	Non-structural proteins 1
NS2	Non-structural proteins 2
^{o}C	Degree Celsius
OD	Optical density
OIE	World Organization for Animal Health (Office International des Epizooties)
ORF	Open reading frame
P.I.	Post inoculation
P/N	Positive-to-negative ratio
PA	Polymerase acidic protein
PB1	Polymerase basic protein 1
PB2	Polymerase basic protein 2
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
pH	Puissance hydrogen (hydrogen-ion concentration)
RBC	Red blood cell
RE	Restriction enzyme

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RNA	Ribonucleic acid
Rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcriptase –polymerase chain reaction
S	Seconds
S/P	Sample-to-positive ratio
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SOB	Super optimal broth
Sp	Specificity
SPF	Specific-pathogen-free
SRH	Single radial hemolysis
Ss	Single stranded
TAE	Tris-acetate-EDTA buffer
Taq	*Thermus aquaticus*
TBS	Tris-buffer saline
TEMED	N,N,N,N-tetramethylethylene diamine
TMB	3,3', 5,5′-tetramethylbenzidine
TTBS	Tween-tris-buffer saline
UK	United kingdom
UPM	Universiti Putra Malaysia
UV	Ultraviolate
v/v	volume/volume

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VN	Virus neutralization
w/v	Weight/volume
WHO	World Health Organization
X-Gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside

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CHAPTER I

INTRODUCTION

1.1 General Background

Equine influenza (EI) is a highly contagious respiratory disease, causing marked economic losses to the equine industry as a result of a long convalescence and high cost of therapy of sick horses. During infection caused by the EI virus, the horse may show the following typical signs: high pyrexia, dyspnea, coughing and serous to mucopurulent nasal discharge as well as myalgia, anorexia and swelling of regional lymph nodes (Radostits *et al.*, 2007; Sellon and Dubey, 2007; Damiani *et al.*, 2008).

Equine influenza virus is a species–type A influenza virus from the *Orthomyxoviridae* family, and is comprise of eight segments of RNA. These RNA segment are coated by nucleoprotein (NP), which along with a complex of polymerase enzymes, is responsible for transcription and replication of the virus within the nucleus of the host cell. The segments are surrounded by matrix protein (MP) and the entire structure is enclosed within a lipid bilayer called the virion envelope. Two major surface glycoprotein's hemagglutinin and neuraminidase, project from the virion envelope (Myers and Wilson, 2006; Radostits *et al.*, 2007).

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The virus is transmitted by aerosol, wind, nose-to-nose contact, and fomites such as tack, grooming equipment, machinery, water, feed, and human contact. The virus is delicate within the environment and easily killed by heat, cold, desiccation, and disinfectants. The incubation period lasts between 1 and 3 days, and shedding of the virus in nasal secretions begins as soon as 24 hours after infection (Myers and Wilson, 2006).

In horses there are two subtypes of influenza A virus. H3N8 (A/equine-2, first recognized in Miami in 1963), which is the most commonly isolated, and subtype H7N7 (A/equine-1, first described in Prague in 1956) which is more rarely isolated. Both subtypes have caused disease (Muller *et al*., 2005). The H3N8 was first documented in 1963 in Florida and subsequently it was described throughout the world. Recent studies of the H3N8 subtype of equine influenza viruses have demonstrated that, starting from the early 1990s, these strains have diverged into two distinct evolutionary lineages designated as European (A/eq/Suffolk/89-like) and American (A/eq/Newmarket/1/93-like) (Martella *et al*., 2007).

Previous studies suggested that using RT-PCR with the single set of primers from the matrix gene proved more sensitive than virus isolation or two different enzyme immunoassays and less prone to contamination

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than nested PCR. Furthermore, these primers were originally designed to detect all known subtypes of influenza A viruses from multiple species (Fouchier *et al*., 2000; Quinlivan *et al*., 2004, 2005).

Protein expression is an extensive process which that begins with transcription, translation, folding, post-translation modification and finally targeting. Protein expression can be achieved basically by expression plasmid that encodes the desired protein, subjecting the recombinant plasmid into the required host cell, growing the host cell and inducing expression, ending with SDS Page analysis to confirm the presence of the desired protein (McFarland and Douglas, 2000; Peter, 2005). *Escherichia coli* remain the most frequently used prokaryotic expression host. *E. coli* is a popular host for a variety of reasons including rapid growth, high expression levels for many recombinant proteins, well characterized genetics, a large number of cloning vectors and mutant host strains. The IPTG inducible T7 RNA polymerase has been extensively used for recombinant protein expression in *E. coli* (Hanning and Makrides, 1998; Baneyx, 1999).

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The *E. coli* expression system is known to be the fastest, easiest and least expensive technique that can be used to express usable amounts of recombinant protein. These characteristics, coupled with a vast amount of knowledge about it and the many years of experience with expression of foreign genes, have established *E. coli* as the leading host organism for most scientific applications in protein expression (Baneyx, 1999).

Non-structural proteins (NS protein) were used for identification and differentiation between vaccinated and naturally infected animals. Liu *et al*. (2003) and Zhao (2004) cloned and expressed the NS1 gene of H9N2 avian influenza virus from chicken and duck. The NS1 has many advantages to be used as differentiation marker between vaccinated and naturally infected animals, these include: 1- NS1 of influenza virus is very conservatives when compared with other proteins, which have poor antigenicity; 2- temporal regulation of viral RNA synthesis; 3- control of viral splicing; 4- enhancement of viral mRNA translation; 5- regulation of virus particle morphogenesis; vi- suppression of host immune/apoptotic responses; and vii- involvement in strain-dependent pathogenesis (Hale *et al*., 2008). Horses that were vaccinated with inactivated vaccines have not produced non-structural protein specific antibodies, while they have presented in the naturally infected horses. This has allowed the possibility

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of differentiation diagnosis between vaccinated and naturally infected horses.

1.2 Problem Statement

The diagnosis of infectious disease has traditionally been done by the direct demonstration and identification of the causative agent by culturing and isolation processes. Unfortunately, this may be beyond the expertise and capabilities of many diagnostic laboratories, particularly in developing countries. However, accurate diagnosis can be achieved when serological techniques are used in combination with the clinical observation and epidemiological history. Although classical serological techniques like agglutination, precipitation, complement fixation and virus neutralisation tests have proved useful, they suffer from number of drawbacks. In general these drawbacks relate to a combination of inadequate diagnostic performance, lack of standardization and poor efficiency. Because enzyme-linked immunosorbent assay (ELISA) potentially resolve all of these problems, currently great deal of emphasis has been placed on research, development, evaluation and application of these techniques in the diagnosis of infectious diseases of veterinary importance (Wright *et al*., 1993; Jacobson, 1996, 2000).

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The ELISA is more preferable for antibody detection because it is economical, reliable, sensitive and able to quickly provide results on large numbers of samples. Commercial ELISA kits are currently available for the detection of antibodies for EIV in field samples, but these kits are based on the use of NP protein, which are produced by conventional technology, as the antigen source. However, there are reports of using recombinant expression products in eukaryotic system such as ELISA antigens and field samples but no report of using recombinant prokaryotic products as ELISA antigen. Molecular tools for detection, and identification of the causative agent for equine influenza virus is very important specially in early stages for the control and reduction the chances of outbreaks, RT-PCR considered as very rapid, accurate feasible tool for EIV detection and identification. Despite the extensive use of vaccines in some horse populations, outbreaks of equine influenza continue to occur. Vaccination programs for the control of equine influenza have limitations due to the problem of differentiating between vaccinated and virus-infected horse antibodies. EIV vaccines available in Malaysia are inactivated vaccines However, this vaccine have a shortcoming of disturbing the monitoring of EI. Repeated vaccinations lead to prolonged high HI titres, which may complicate the classical serological diagnosis (four-fold antibody titre increase). Antibodies to NS1 protein are only present after viral infection; therefore vaccinated animals should be devoid of such antibodies. From

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practical point of view this is very useful when only single serum samples are available from convalescent animals for testing (lack of paired sera in imported animals during quarantine).

1.3 Significance of the Study

Rapid diagnosis and isolation of affected horses are the front lines of defence against outbreaks. Traditionally, the gold standard for the diagnostic method of EIV has been virus isolation from nasopharyngeal swabs using embryonated hen's eggs and/ or Madin-Darby Canine kidney (MDCK) (Yamanaka *et al*., 2007; Myers and Wilson, 2006). With the advent in molecular biology based technique, reverse transcription polymerase chain reaction (RT-PCR) can be a valuable tool in the surveillance of equine influenza virus (Oxburgh and Hagstrom, 1999).

EIV is considered as very important disease for public health because horses are regarded as 'mixing vessels' for avian viruses and human influenza viruses. Therefore, it is vital to establish a method to distinguish between vaccinated and naturally infected horses. In addition, information for ELISA test based on prokaryotic recombinant NS1 protein of EIV has not been reported so far. In Malaysia, there were no reports on the

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practical use of NS protein to distinguish vaccinated and naturally infected horses. In this study, the NS1 gene of H3N8 subtype equine influenza A virus was cloned, expressed, and purified. The antigenicity was analyzed. This study considered as a basic study for developing an effective diagnostic method for rapid detection of equine influenza virus.

1.4 Hypotheses

The hypotheses for this study that will be tested are:

1- EIV is present among horse population in peninsular Malaysia.
2- EIV can be isolated from nasopharyngeal swabs from horses by SPF chicken embryonated eggs.
3- NS1 protein can be express in prokaryotic expression system.
4- NS1 protein can be used as a marker for differentiation between vaccinated and infected horses with EIV.

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1.5 Objectives

The general objectives of the present study were:

1- To detect, identify and isolate the equine influenza virus from field nasopharyngeal swabs from unvaccinated horses using matrix gene (M) and haemagglutinin gene (H3) primers by reverse transcriptase polymerase chain reaction (RT-PCR) and specific-free-pathogen (SPF) embryonated eggs for virus isolation.

2- To clone, express, and purify the EIV non-structural (NS1) protein of strain H3N8 in prokaryotic expression system.

3- To develop and optimize the working conditions of an indirect ELISA kit based on recombinant NS1 protein

4- To validate and evaluate of the antigenic properties and diagnostic potential of the expressed NS1 protein for detection and differentiation between vaccinated and natural infected horses.

5- To compare the performance of the developed ELISA with an indirect equine influenza IgG-ELISA "IBL-ELISA" and competitive equine influenza "IDEXX-ELISA".

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