Chapter I

Introduction

Horses, one of the most historically vital domesticated animals to humans, have played a central role in the lives of people for tens of thousands of years. No other animal, domestic or wild, has had so great an impact on the history of civilization as has the horse. The horse was an integral element in warfare and conquest, in transportation and travel, and in art and sport. Its beauty and power are legendary. Since ancient times, the horse has been depicted and revered as the noble bearer of heroes, champions, and gods.

In its design, form, and function, the horse is superbly suited as a purely riding animal. Its spine is fixed and rigid and well devised to bear weight. Its stature is tall, a feature that lends any rider a towering advantage in hunting, sport, and warfare. The horse's legs are long, slender, graceful, and, above all, swift. The speed and ability to cover ground has made horses invaluable to people, and remains so today **(International Commission on Zoological Nomenclature, 2003)**.

Horses are highly social and intelligent herd animals. They fail to thrive in isolation. Their intrinsic nature is to seek a relationship, which offers mutual benefit, ensuring kinship and protection. Humans have long exploited this social character to their advantage. Through domestication the horse's instinct to herd with

other horses has become one to "herd" with or embrace people, and the resulting "willingness to please" makes horses valuable to humanity and heroic figures in movies and literature **(Siegal, 1996)**.

The Arabian horse, the world's oldest purebred breed, gained the title of a "hot blood" for its temperament. Arabians are valued for their sensitivity, keen awareness, athleticism, and energy. Combined with the lighter, refined bone structure, they were used as the foundation of the thoroughbred, another "hot blood."

The thoroughbred is unique to all breeds in that its muscles can be trained for either fast-twitch (for sprinting) or slow-twitch (for endurance) making them an extremely versatile breed. Another famous modern breed of hot blood is the American Quarter Horse. The most popular breed in the United States, it is commonly believed to be the world's fastest horse, some having been clocked at 55 mph at the finish line in racing events. True hot bloods usually offer greater riding rewards than do other horses. Their sensitivity and intelligence enable quick learning and greater communication and cooperation with their riders **(Riegal *et al*, 1996)**.

Muscular and heavy draft horses are known as "cold bloods," as they have been bred to be workhorses and carriage horses with calm temperaments. Draft horses originate mainly from northern Europe, and especially from Great Britain. Harnessing a horse to a carriage requires some level of trust in the horse to remain calm when restrained. The best known cold bloods would probably be the Budweiser Clydesdales, a breed that comes from Scotland.

"Warm blood" breeds began in much the same way as the thoroughbred. The best of their carriage or cavalry horses were bred to Arabian, Anglo-Arabian, and thoroughbred sires. The term "warm blood" is sometimes used to mean any draft/thoroughbred cross. Currently, the warm blood name has become the term to specifically refer to the sport horse breed registries that began in Europe. These registries, or societies have dominated the Olympics and World Equestrian Games in Dressage (competitive horse training) and Show Jumping since the 1980s **(Riegal *et al*, 1996)**.

Influenza A virus infection of equips has been reported worldwide with the exception of a small number of island countries including New Zealand and Iceland. Equine influenza EI is endemic in Europe and America. Other parts of the world such as Japan, South Africa, India and Hong Kong suffer occasional incursions but the disease is not endemic. Typical outbreaks of EI are characterized by pyrexia, coughing and nasal discharge. Although the mortality rate associated with equine influenza virus EIV infection is very low it is considered the most important respiratory virus of horses. This is because it is highly contagious and has the potential to cause significant economic loss due to the disruption of major equestrian events. The equine population is highly mobile and horses travel long distances by road and air for competition and breeding purposes. When an infected horse is introduced into a susceptible population, virus spread can be explosive. The incubation period can be less than 24 hours in naïve horses and the continuous coughing which is a major feature of the disease, serves to release large quantities of virus into the environment. The virus is spread by the respiratory route, by personnel, vehicles contaminated with virus, and by fomites. Large outbreaks are often associated with high density stabling, the congregation of horses at equestrian

events and their dispersal over a wide geographic area after the event **(Callinan, 2008)**.

The clinical signs are less severe and disease spread is slower in partially immune populations. The majority of outbreaks in endemic populations are contained with limited spread between premises. The severity of the disease depends primarily on the immune status of the horses at the time of exposure, the environment and the stress created by continuing to work or train. EI can be controlled by vaccination and antibodies against the virus haemaglutinin HA induced by inactivated and sub unit vaccines correlate with protection. In endemic countries the economic losses due to EI can be minimized by vaccination of highly mobile horses and many racing authorities and equestrian bodies have mandatory vaccination policies that serve as an insurance for business continuity. In well vaccinated race horses or competition horses the predominant sign may be sub-optimal performance and many horses may be sub clinically infected. Non-endemic countries rely heavily on vaccination of imported horses to help prevent an incursion. However sub clinically infected vaccinated horses can shed virus. Many countries have experienced EI epizootics related to the importation of such horses **(Daly *et al*., 2006 and United states department of agriculture, October 2001)**.

To-date only two stable subtypes of EI have been reported in horses, H7N7 and H3N8 **(Madic *et al*., 1996 and Webster, 1993),** The first reported outbreak of equine respiratory disease to be confirmed as equine influenza occurred in 1956 in Eastern Europe. The virus isolated was characterised as H7N7. Subsequently H7N7 viruses were identified as the cause of outbreaks in Europe, Asia and the United States. Phylogenetic analysis of these viruses, indicates that they are the most

ancient of all mammalian influenza virus lineages. Although H7N7 viruses co-circulated with H3N8 viruses in horses for many years, it is generally accepted that these viruses have not been active for a long period and may be extinct. Phylogenetic analysis of nucleoprotein genes suggest that the H3N8 equine 2 virus genome originated in the late 19th Century but the first isolation of a virus of this subtype took place in Florida in 1963. Since then H3N8 influenza viruses have been responsible for epizootics in all continents. Antigenic drift occurs less frequently in equine influenza viruses than in human viruses but the H3N8 subtype has evolved into two distinct lineages designated the "American-like" lineage and the "European-like" lineage based on the initial geographical distribution of viruses. Three American sub-lineages subsequently emerged in Argentina, Kentucky and Florida. The Florida sub-lineage has more recently diverged into two Clades; Clade 1 includes the viruses A/equine/South Africa/4/2003, A/equine/Sydney/2007 and A/equine/Ibaraki/2007 responsible for the epizootics in South Africa, Australia and Japan respectively, and Clade 2 includes A/equine/ Newmarket /03 and other viruses that have been circulating in Europe since 2003. Antigenic drift of the H3N8 viruses impacts on vaccine efficacy. This has been demonstrated repeatedly in the field. Since the introduction of mandatory vaccination in the UK in 1981 there have only been two large outbreaks of EI in 1989 and in 2003 and in both instances the vaccine strains had been isolated ten years earlier **(Daly *et al.*, 2004 and Lamb & Krug, 2001)**.

Influenza virus reassortants originating from avian, human and/or swine viruses have not been identified in horses and to-date the epidemiology of EI appears to be somewhat less complex than that of swine or avian viruses. The current EI viruses are believed to be of avian ancestry and more recent transmission

of avian viruses to horses and donkeys has been recorded **(Lamb & Krug, 2001)**. The sequence analysis of an H3N8 virus isolated in 1989 from horses during an influenza epidemic in North Eastern China established that the virus was more closely related to avian influenza viruses than to equine influenza viruses. It was reported that over 13,000 horses were affected and that the mortality rate was over 20% but the virus did not persist and failed to spread beyond China. More recently avian H5N1 has been associated with respiratory disease in donkeys in Egypt **(Lamb & Krug, 2001)**.

Equine influenza EI viruses have the potential to cross species barriers and have been associated with outbreaks of respiratory disease in dogs (primarily but not exclusively, greyhounds and dogs in shelters) in North America, quarry hounds in England and dogs on premises with horses affected by EI in Australia. Interspecies transmission of EIV to dogs maintained in the same stable as experimentally infected horses was demonstrated but there is to-date no evidence of EI transmission from dogs to horses. During 2004-2006 swine influenza surveillance in central China 2 equine H3N8 influenza viruses were isolated from pigs. Despite the successful experimental infection of human volunteers with EIV and the occasional identification of seropositive persons with occupational exposure there is currently little evidence of zoonotic infection of people with EI **(Callinan, 2008 and Lamb & Krug, 2001)**.

Main objectives for influenza virus surveillance in horses

1. To monitor genetic and antigenic evolution of EI.

2. To monitor vaccine efficacy.

3. To serve as an early warning for veterinarians and horse owners, facilitating the implementation of appropriate prophylactic and control measures.

4. To reduce the economic impact of EI by maintaining awareness of emergence and international spread of antigenic variants.

Chapter II

Literature Review

2.1 Virus classification

Influenza viruses belong to the family of *Orthomyxoviridae* that contains 5 genera: influenza A, B, and C viruses, Thogotovirus (sometimes called influenza D, tick borne virus) and Isavirus (Infectious salmon anemia virus) **(Morse *et al.*, 1992, Kawaoka *et al.*, 2005 and Kibenge *et al.*, 2008)**.

The name *Orthomyxoviridae* originated from the ability of such viruses to specifically bind to mucus (hemagglutination) and distinguish it from another family of enveloped negative-stranded RNA viruses *(Paramyxoviridae)* **(Cox *et al.*, 2000 and Lamb and Krug, 2001)** The influenza A, B, and C viruses can be distinguished on the basis of morphological features and antigenic differences among their (nucleocapsid) NP and (matrix) M proteins **(Lamb and Krug, 2001 and Kollerova and Betáková, 2006)**.

Thus influenza "A" viruses can be classified according to:

A) Structure

Variations in the antigenicity of the HA and NA allowing to classify influenza "A" viruses into 16 different (hemagglutinin) HA subtypes (H1 to H16) and nine different (neuraminidase) NA subtypes (N1 to N9) **(Fouchier *et al.*, 2005)**.

B) Virulence

Based on their virulence, AI viruses are categorized into highly pathogenic HP and low pathogenic LP. The amino acid changes at the HPAI viruses HA cleavage site **(Senne *et al.*, 1996, Horimoto and Kawaoka, 2001 and Olsen *et al.*, 2006)** particularly the presence of additional multiple basic amino acids **(Wood *et al.*, 1994, Perdue *et al.*, 1997 and Zambon and Fleming, 2001)** facilitate replication and as a result outbreaks by such viruses cause high mortalities approaching 100% and are limited to the H5 and H7 subtypes **(Bosch *et al.*, 1979)**.

C) Genetic Characterization

Recent genetic characterization of H5N1 viruses has demonstrated two distinct phylogenetic clades **(Webster *et al.*, 2006)**. Clade 1 that comprises the circulated viruses in Cambodia, Thailand, and Vietnam, while clade 2 comprises the circulated viruses in China and Indonesia that spread towards the Middle East, Europe, and Africa **(Chutinimitkul *et al.*, 2007)**. Clade 2 could be further classified into several subclades three of which are 2.1, 2.2 and 2.3 that vary in their geographical distribution were responsible for human cases and particularly viruses belonging to subclade 2.2 caused outbreaks among Egyptian poultry associated with human infections **(Smith *et al.*, 2009)**.

2.2 Virus composition

Influenza viruses are enveloped viruses with a segmented negative sense single stranded RNA (ssRNA), belonging to the family Orthomyxoviridae. The Orthomyxoviridae viruses are composed of about 1% RNA, 70% protein, 20% lipid, and 5% to 8% carbohydrate. This family is classified into 5 groups including

influenza viruses' type A, type B, type C, Thogotovirus, and Isavirus (**Cox *et al.*, 2000, Lamb and Krug, 2001 and Kawaoka and Noda, 2006**). The lipid envelope of influenza viruses is derived from the plasma membrane of the host cell in which the virus is grown. Influenza viruses' type A, B and type C differ in the antigenicity of their nucleoprotein and matrix proteins (**Lamb and Krug, 2001**).

All members of the genus influenza "A" viruses have eight single-stranded negative-sense RNA genome segments encoding 11 proteins (Fig. 2-1). On the basis of the gene sequences for influenza "A" virus, the segments 1-3 encode PB1, PB2 and PA polymerases and estimated to be 87 kDa, 84 kDa and 83 kDa respectively. The segment 4 encoding HA is 63 kDa (HA_1 is 48 kDa and HA_2 is 29 kDa). The segment 5 encoding NP is 56 kDa. The segment 6 encoding NA is 50 kDa. The segment 7 encoding M_1 and M_2 proteins are 28 kDa and 11 kDa respectively. The segment 8 encoding NS_1 and NS_2 proteins are 27 kDa , 14 kDa respectively (**Lamb and Krug, 2001)**. PB1-F2 is the most recently discovered influenza "A" virus protein and contributes to viral pathogenesis (**Zamarin *et al.*, 2006 and Zell *et al.*, 2006**).

Haemagglutinin HA, the major neutralizing and protective surface antigen of influenza viruses, is responsible for influenza virus entry into host cells by attaching to sialic acid receptors on host cell surface. Subsequently, fusion of virus with host membrane in the endosome after the virus has been taken up by endocytosis which results in the intracellular release of the virion contents (**Palese and Shaw, 2006).** The hemagglutination activity of HA surface protein of influenza viruses was used for concentration and detection of very low number of influenza viruses in large volume of water (**Khalenkov *et al.*, 2008**).

HA, synthesized as an HA0 precursor (noncovalently bound homotrimers) on the viral surface is required to be cleaved by host proteases at a conserved arginine residue to HA1 and HA2, for productive infection (**Steinhauer, 1999**). Many studies revealed that the difference in pathogenicity between human influenza and avian influenza strains correlates with structural difference at the HA0 cleavage site and the specificity of proteases involved in this cleavage (**Horimoto and Kawaoka, 1994 and Decha *et al.*, 2008**).

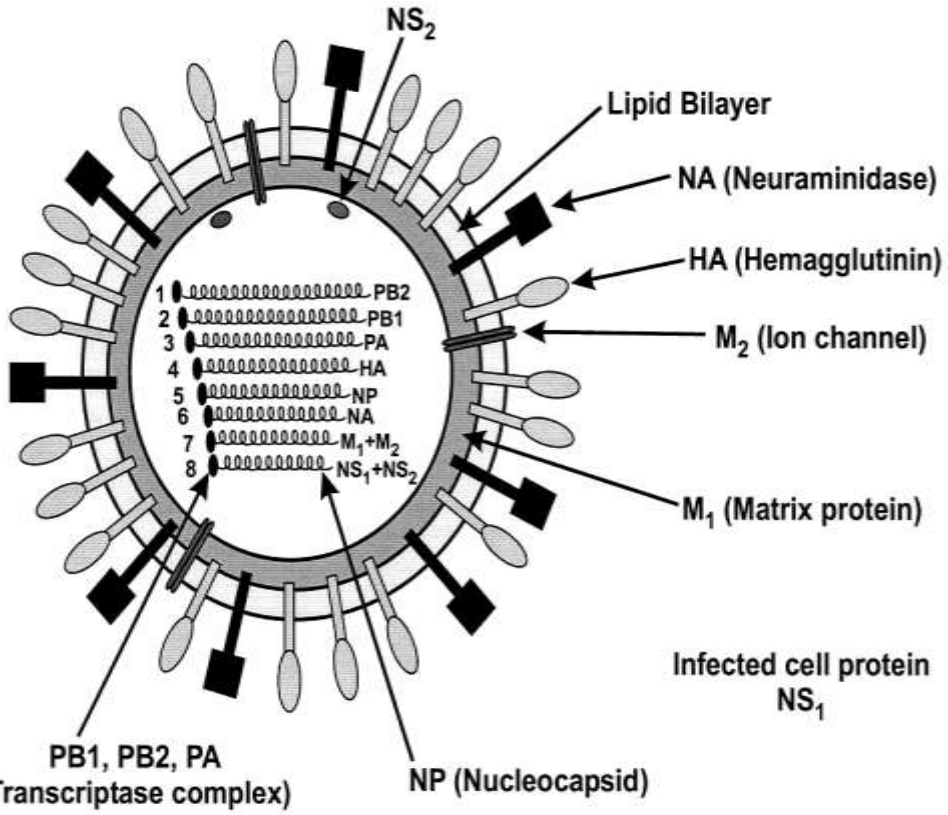

Figure 2-1. Schematic diagram of the structure of the influenza A virus particle obtained from Lamb and Krug (2001).

The proteases responsible for cleavage of HA0 in human influenza infections are secreted by either cells of the respiratory tract by Clara cells of the bronchiolar epithelium, co-infecting bacteria or may be produced in inflammatory responses to infection. These proteases limit the efficiency of viral infectivity and its ability to spread beyond the respiratory tract causing systemic infection (**Webby *et al.*, 2004)**. In case of the highly pathogenic avian influenza strains HPAI, the multi-basic cleavage sites of HA0 are cleaved by widely distributed host intracellular proteases, such as furin and other subtilisin-like enzymes. Such enzymes are involved in the post translational processing of hormone and growth factor precursors, resulting in systemic infections (**Guo *et al.*, 2008**).

(Sasan *et al.* 2008) demonstrated the multi basic cleavage sites of some HPAI, for example the H5N1 strains including multibasic cleavage site (PQGKRRRKKR*GLF) of 2 Egyptian strains of the same virus H5N1 isolated from goose and duck. The 1918 H1N1 virus did not have a multi-basic cleavage site of the HA0 precursor, indicating that this feature is not the only requirement for a strain to be virulent in humans (**Li *et al.*, 2004**). The neuraminidase enzyme, the second neutralizing surface antigen of influenza, is involved in the release and spread of mature virions that have successfully multiplied in host cells by cleaving sialic acid residues that bind the mature viral particles to the infected host cells (**Matrosovich *et al.*, 2004**). Matrix proteins, M1 and M2, are generated by RNA splicing. M1 is entirely internal and located immediately below the lipid bilayer of the virus, while M2 serves as an ion channel that has a small extracellular surface domain (**Aoki, 1998**). The nonstructural NS proteins are NS_1 and NS_2 or nuclear export protein NEP. The NS_1 is the only nonstructural protein that is not packaged into the virion (**Krug *et al.*, 2003**).

The NS_1 protein is a regulator of both mRNA splicing and translation. It also plays a critical role in counteracting the type I interferon IFN, which is the antiviral immune defense of the host cells. Thus, NS_1 protein is directly related to the pathogenicity of the influenza virus and might be a target for vaccine development (**Li *et al.*, 2006 and Palese and shaw, 2006)**. The NS_2 protein, known as the nuclear export protein NEP is present in small amounts in the virions in association with the ribonucleoprotein RNP through interaction with the M1 protein. The role of NS2 protein is mediating the export of newly synthesized RNPs from the nucleus and it plays a role in viral assembly (**Lee and Saif, 2008**).

The influenza A virus ribonucleoprotein complex consists of four proteins necessary for virus replication: PB2, PB1, PA and NP. The nucleoprotein NP is an important protein in differential diagnosis of influenza A, B and C. This protein is mainly responsible for encapsulation of the genomic RNA segments (**Lamb and Krug, 2001**). The remaining three internal proteins, namely PB1, PB2, and PA polymerases are involved in viral gene transcription. The PB1 subunit is responsible for the polymerase and endonuclease activities (catalyzes the sequential addition of nucleotides during RNA chain elongation). PB2 binds the cap structure of cellular pre-mRNAs and PA is a phosphoprotein with protease activity involved in RNA replication (**Resa-Infante *et al.*, 2008**).

Undoubtedly, mutations in other viral proteins, including PB2 **(Hatta *et al.*, 2001)** and NS1 **(Seo *et al.*, 2002 and Geiss *et al.*, 2002)**, which might confer efficient avian-virus replication in humans, might also be required.

The 11^{th} influenza A virus gene product is an 87-amino-acid protein provisionally named PB1-F2 because it is encoded by an open reading frame

overlapping the PB1 open reading frame. A significant fraction of PB1-F2 localizes to the inner mitochondrial membrane in influenza A virus-infected cells. PB1-F2 affects apoptosis and may contribute to the pathogenicity and lethality of influenza A viruses (**Gocnikova and Russ, 2007)**.

2.3 Stages of replication of influenza viruses

During the infectious cycle, influenza viruses bind to cell surface sialic acid through the receptor-binding site in the distal tip of the HA molecules **(Skehel and Wiley, 2000)**. Different influenza viruses have different specificities for sialic acid linked to galactose by α 2,3 or α 2,6 linkages, and this is dependent on specific residues in the HA receptor-binding pocket **(Matrosovich and Klenk, 2003 and Gambaryan *et al.*, 2005)**. After attachment, the virus is taken up by the cell via a clathrin-coated receptor-mediated endocytosis process **(Matlin *et al.*, 1981)**.

While migrating to the cytosol, vRNPs have to cross the membranes of both the virions and endosomes **(Lamb and Krug, 2001**). This is accomplished by HA-mediated fusion of the viral membrane with the cellular membrane as shown in (Fig. 2-2) **(White *et al.*, 1981 and Steinhauer *et al.*, 2001)**. The M2 ion channel plays an important role in viral uncoating process and is thought to facilitate the flow of protons from the lumen of the endosome into interior part of the viral particle. This acidification of the endosome causes conformational changes in HA that result in fusion peptide insertion into the target endosomal membrane and close proximity of host endosomal and viral membranes **(Steinhauer *et al.*, 2001**).

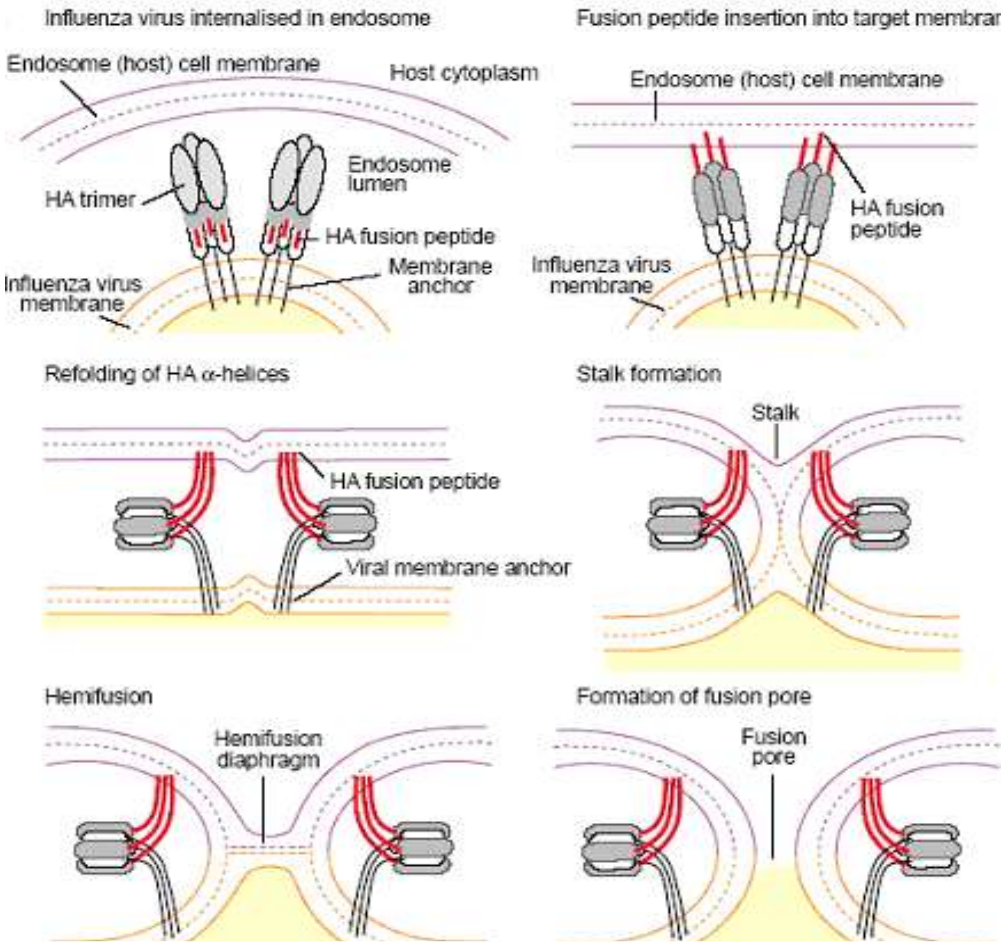

Figure 2.2. Schematic representation of fusion between viral and cellular membrane (Steinhauer *et al.,* 2001).

Further refolding of the C-terminal region of HA and clustering of HA trimers lead to bending of the two membranes towards each other **(Chen *et al.*, 1999)**. The outer lipid bilayer leaflets of the two membranes are pulled together to form a highly bent stalk structure, leading to formation of the hemifusion intermediate structure **(Steinhauer *et al.*, 2001)**. In the hemifusion intermediate, the distal leaflets are pulled towards each other to form a dimple, and the hemifusion diaphragm continues to expand as a result of the bending tension of the HA molecules and eventually breaks **(Chen *et al.*, 1999)**. This results in the formation of a fusion pore that flickers then dilates, allowing mixing of virus interior contents with the cytoplasm and ultimately import of viral nucleocapsids into the nucleus **(Neumann *et al.*, 2000; Whittaker, 2001 and Colman and Lawrence, 2003)**.

Unlike other RNA viruses, influenza mRNA synthesis has a unique dependence on host cell nuclear function. In the nucleus, viral ribonucleoprotein vRNP undergoes transcription (mRNA synthesis) and replication (formation of positive-sense complementary RNA that replicates to produce vRNA [minus strands] followed by vRNP synthesis) **(Elton *et al.*, 2002)**. Influenza NP is a multifunctional RNA-binding protein involved in switch from mRNA to template RNA synthesis and in virion RNA synthesis **(Portela and Digard, 2002)**.

The virus trans-membrane TM proteins (HA, NA and M2) are synthesized on membrane-bound ribosomes and translocated across the membrane of the endoplasmic reticulum ER in a signal recognition particle dependent manner **(Lamb and Krug, 2001)**. In the ER, N-linked carbohydrate chains are transferred to HA and NA from a dolicyl lipid carrier or by trimming of terminal glucose residues from mannose-rich oligosaccharides occurs, proteins are transported out of

the ER to the golgi apparatus. In HAs containing a furin cleavage site, activation occurs by cleavage in the trans golgi network TGN **(Wright and Webster, 2001)**.

(Nayak *et al.* 2004) reviewed assembly and budding of influenza virus (Fig. 2-3) as following. Both HA and NA from Golgi and the trans Golgi network TGN by the exocytic pathway to the specific assembly site, budding site containing lipid rafts, on the plasma membrane. Another glycoprotein M2 is transported via the same route but does not require lipid rafts. Viral interior containing genomic RNA, NP, NS, 3P and M1 are transported to the assembly site on the plasma membrane either via cytoskeleton elements or by piggy-backing on the cytoplasmic tail of HA and NA. Ml binds on its outer side to the cytoplasmic tails of both HA and NA-TM domains and on its inner side to the vRNP. Finally, the plasma membrane bends at the assembly site containing glycoproteins and the Ml–vRNP complexes, causing an outward membrane curvature. Eventually, fusion of the apposing cellular and viral membranes leads to fission and pinching-off of the virus particle, releasing the enveloped progeny virus particle into the extracellular environment. Influenza virus requires NA to release virus particles from sialic acid residues on the cell surface and spread from cell to cell.

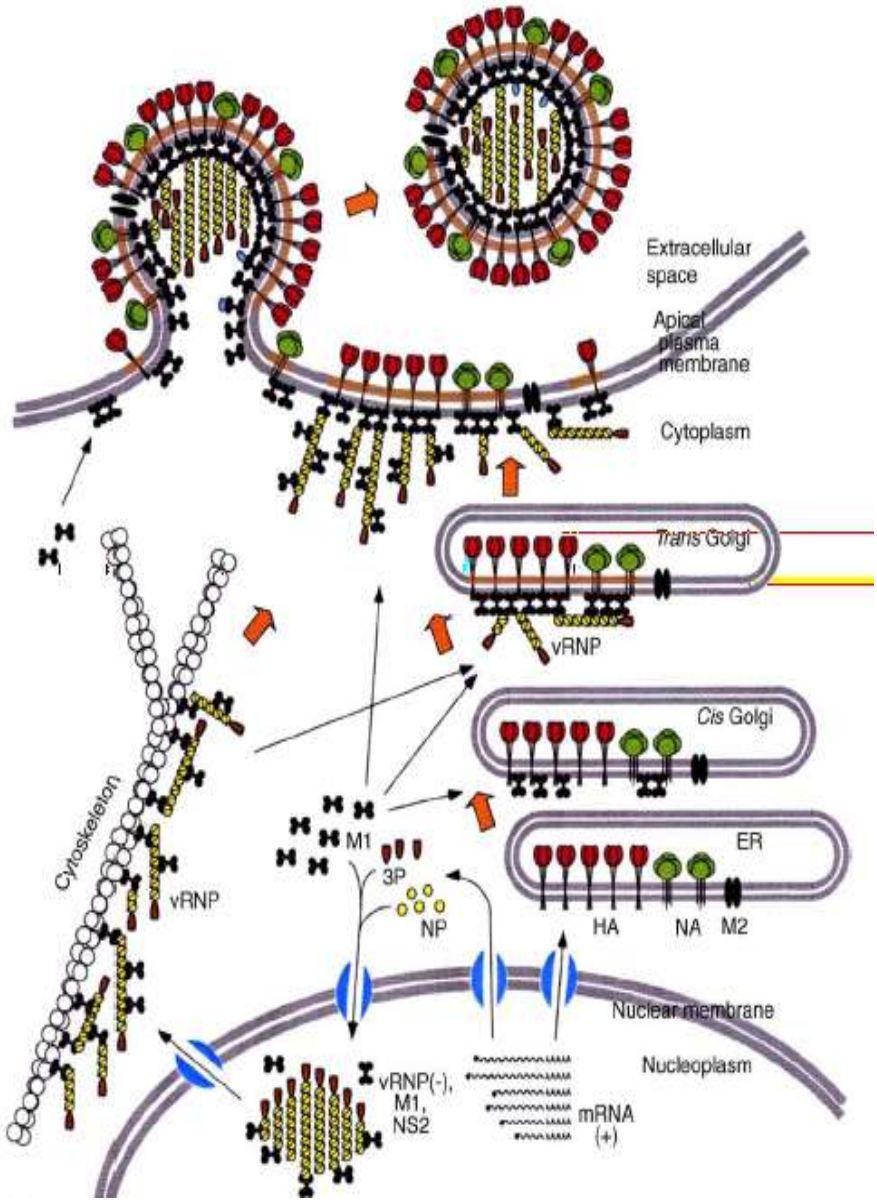

Figure 2.3. Schematic representation demonstrating assembly and budding of influenza virus (Nayak *et al.,* 2004).

2.4 Epidemiology of Influenza Viruses in Horses

2.4.1 Equine influenza subtypes (EIV)

Influenza A viruses are subtyped according to the antigenicity of HA and NA. Currently, 16 HA and 9 NA subtypes have been identified among influenza A viruses **(Fouchier *et al*, 2005)** All these subtypes are found in avian species. However, influenza viruses infecting mammals are limited to a few subtypes **(Horimoto and Kawaoka, 2001)**.

Two equine influenza virus subtypes have been recognised, the H7N7 subtype equi1 and the H3N8 subtype equi2. Due to genome segmentation, genetic reassortment can occur in cells simultaneously infected with two different subtypes of influenza A virus. Reassortment of the HA or NA genes is the basis for antigenic shift but this has not been observed in horses **(Ito *et al*, 2008)**.

Equine influenza was first recognised in 1956, when influenza was recovered during a widespread epidemic of respiratory disease among horses in Eastern Europe as shown table 2-1, **(Sovinova *et al*, 1958)**.

The virus (A/eq/Prague/56), which has an H7 haemagglutinin HA and an N7 neuraminidase NA, was designated as the prototype equine influenza virus, historically referred to as equine subtype 1. The last confirmed outbreak caused by an H7N7 subtype virus was in 1979; however H7- specific antibody has been reported in horses believed to be unvaccinated, suggesting that the virus may still circulate in a subclinical form.

In 1963, an equine influenza virus of a different antigenic subtype H3N8 originally designated as equine subtype 2, caused a major epidemic in the USA

(Waddell *et al*, 1963). The prototype virus, A/eq/Miami/63, was introduced into the equine population of Florida with the importation of horses from Argentina **(Scholtens and Steele, 1964)**. Field evidence suggested that regular vaccination provided protection against H7N7 infections, but that the H3N8 component of the vaccine was less effective. For example, in January 1976 a localised outbreak of H3N8 occurred in Thoroughbred horses in Newmarket (UK) at a time when many animals had recently been vaccinated **(Thomson *et al*, 1977)**.

Clinical influenza affected unvaccinated and some vaccinated horses, with the severity of disease corresponding with the period since vaccination. Stables in which over 75% of horses were vaccinated were not affected seriously. Between 1978 and 1981, widespread epidemics of H3N8 viruses were reported in Europe and North America with infections occurring in vaccinated as well as unvaccinated horses. In Britain in 1979, influenza was confined to unvaccinated horses during the first six months of the year, but spread to vaccinated Thoroughbreds in June 1979, providing clear evidence that the vaccines did not provide immunity against field infection for the full year between "booster doses" **(Burrows and Denyer, 1982)**. Racing was affected, and this led to the subsequent introduction of mandatory vaccination in the UK and Ireland in 1981.

In 1989, there was again a major epidemic of influenza H3N8 in Europe affecting not only unvaccinated but also large numbers of vaccinated horses **(Livesay *et al*, 1993)**. This represented the first major outbreak in Britain since 1979.

Outbreaks of equine influenza have occurred sporadically in Europe and on the American continent since 1989 epidemic. In the last 15 years, there have also

been a number of serious outbreaks of H3N8 influenza in populations with no previous history of the disease. In 1986 and 1987, the infection was introduced into South Africa and India, respectively. The source of these outbreaks could be traced to the transportation of infected horses by air from areas where influenza was endemic. Inadequate quarantine at the port of entry allowed the introduction of infected horses into the local susceptible populations with subsequent explosive spread of disease and some mortality. Analysis of the HA genes of the South African and Indian viruses have confirmed their close relationship to viruses circulating in the USA and Europe at the time. In 1989, an influenza epidemic was reported in horses in China with morbidity rates as high as 80% and mortality rates reaching 20% in some herds. Fatal cases were always associated with bacterial infection **(Guo *et al*, 1991)**.

The origin of this outbreak was not traced to the importation of equidae and indeed the antigenic characteristics of this virus appear markedly different from other equine H3N8 isolates. On the basis of sequence information, it was proposed that this virus was derived from an avian source and as such represented a new interspecies transmission event. Although this avian derived virus successfully transmitted to horses and lost its ability to infect ducks, it did not spread beyond China and did not persist in the local horse population beyond 1990. Further outbreaks in Hong Kong in 1992, Dubai in 1995, and the Philippines in 1997 highlighted the ease with which equine influenza outbreaks can be introduced into susceptible populations as a result of international movement of horses (Tab 2-1) **(Callinan, 2008)**.

Table 2-1: **Influenza A virus subtypes isolated from horses, in chronological order of first isolation**

Subtype	Lineage	First isolation	Location	Epidemiology
H7N7	Equine-1 influenza viruses	1956	Worldwide	Low-level circulation or extinct
H3N8	Equine-2 influenza viruses Two lineages (American and Eurasian	1963	Worldwide	Endemic
H3N8	Avian-like viruses	1989	Asia	Two epidemics and evidence of continued exposure

(Guo *et al*, 1992; Paillot *et al*, 2006)

2.4.2 Evolution of equine influenza viruses

Phylogenetic analysis of HA sequences revealed that equine H3N8 viruses, which had been evolving as a single lineage for at least two decades **(Kawaoka *et al*., 1989)**, diverged into two distinct lineages during the mid-1980s **(Daly *et al*., 1996)**. Viruses in one lineage were predominantly isolated from horses on the continent of America, while viruses in the other lineage were almost exclusively isolated from horses in Europe and Asia. The most recent phylogenetic tree of equine H3N8 strains is more complex (Fig. 2-4). The 'Eurasian lineage' strains (shown in green in Fig. 2-4), represented by Newmarket/2/93, continue to form a single clade, but have rarely been isolated in recent years **(Bryant *et al*., 2009)**. The 'American lineage' strains currently predominate, but the lineage has evolved into three distinct clades. The original American lineage strains, represented by Newmarket/1/93 and Kentucky/ 1994 (red in Fig. 2-4), have not been completely

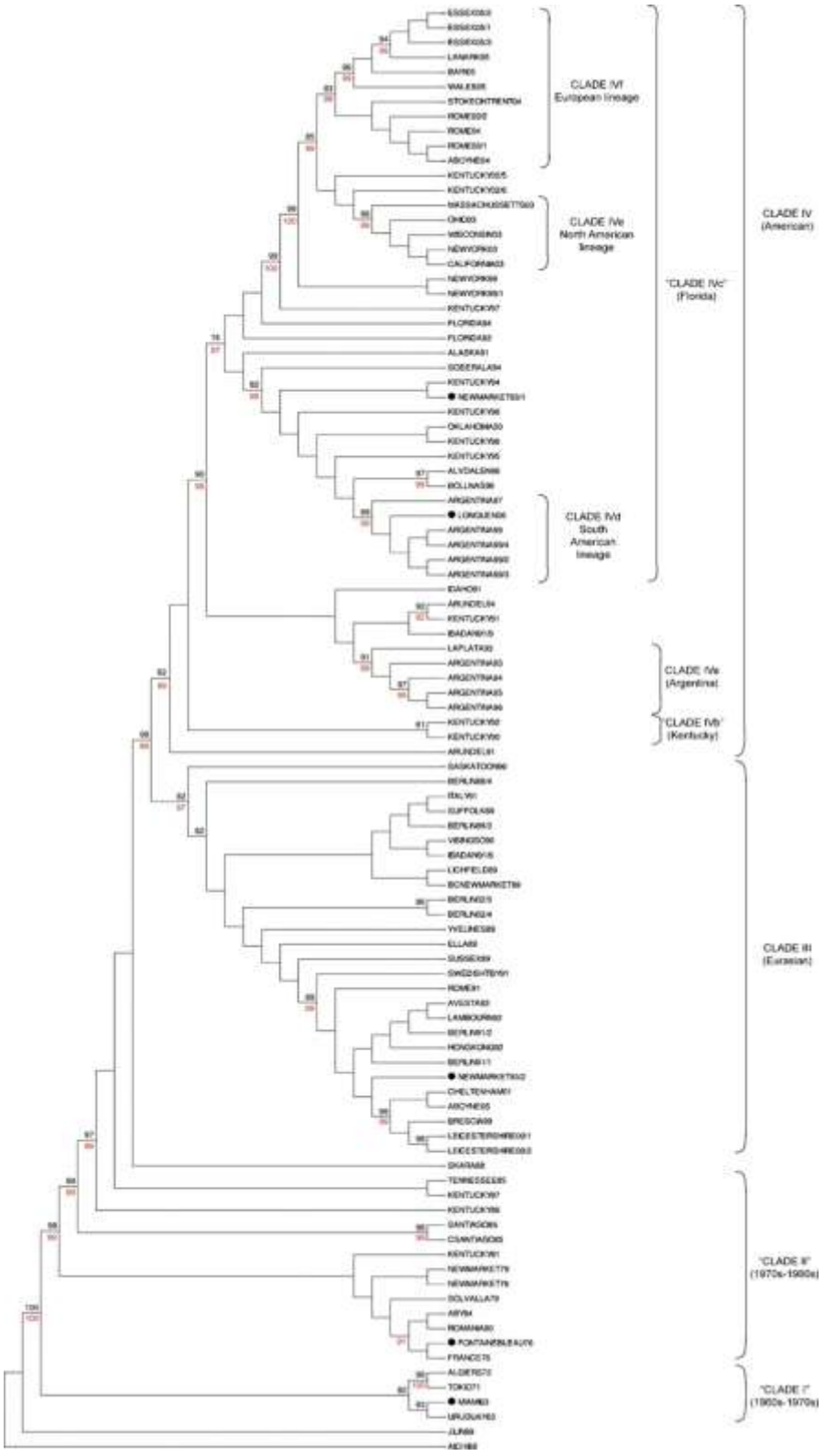

Fig. 2-4 Neighbor joining phylogenetic tree representing evolutionary relations among isolates of the equine influenza virus. Analysis of the HA nucleotide sequence of the H3N8 subtype*: H3N8 prototypes. Clades I to IV and lineages were designated by Lai et al. (2001). Subclades IVa–IVc was designated by Lai et al. (2004). Subclades IVd–IVf designated in this paper correspond of isolate of the 2000 decade. Bootstrap percentages higher than 70% and posterior probabilities above 90% are marked above (in black) and below (in red) branches, respectively. The clades, between quotation marks, correspond to the previous study, which, in this study, do not represent amonophyletic group. The tree was rooted to the A/Aichi/2/1968 (H3N2) isolate.

superseded, with isolations of strains from this clade in the UK **(Bryant *et al*., 2009)** and Chile **(Muller *et al*., 2009)** in 2006.

Within the American lineage is a variant sub-lineage known as the Florida lineage (blue and purple in Fig. 2-4). The strains that gave rise to the outbreak in Newmarket in 2003 and spread across Europe shortly thereafter belonged to this Florida sub-lineage, as have the majority of strains isolated in Europe since 2003 **(Damiani *et al*., 2008; Bryant *et al*., 2009; Rozek *et al*., 2009)**. However, two clades can now be distinguished in this sub-lineage; Florida Clade1 viruses have been isolated in North America since 2003 (e.g., Ohio/2003) and are distinct from the Florida Clade 2 strains that spread to Europe (e.g., Newmarket/5/03). Florida Clade1 viruses caused outbreaks in South Africa at the end of 2003 and subsequently in Japan and Australia in 2007 **(Bryant *et al*., 2009)**. Florida Clade 2 viruses were responsible for the major outbreaks in China, Mongolia and India from 2007 to 2009 **(Qi *et al*., 2010; Virmani *et al*., 2010)**. The phylogenetic analysis points to sporadic incursions of virus from North America into Europe and other regions, as happened around 1993 and 2003, followed by a period of more localized divergent evolution.

2.4.3 Interspecies transmission

Influenza A viruses are known to have evolved from an ancestral avian precursor into different lineages infecting specific species, including horses **(Horimoto *et al*., 2001)**. In 1989, an equine influenza epidemic was reported in north-eastern China and resulted in up to 20% mortality in some herds. The virus causing this outbreak (A/eq/Jilin/89) was shown to be more closely related to avian H3N8 influenza virus than to contemporary equine influenza virus **(Guo *et al*.,**

1992). Despite a successful cross-species transmission, this strain did not spread beyond or persist in China. However, this outbreak illustrates the role of birds, as a natural reservoir of influenza A virus, in the potential emergence of new strains of influenza virus able to infect mammals. Mammal-to mammal transmission of influenza A virus also occurs (e.g. swine to human). In recent years, influenza viruses isolated from dogs (e.g. greyhounds) suffering severe respiratory disease with a high percentage of mortality, were identified as closely related to equine influenza virus H3N8 and could indicate a new case of inter-species transmission (Fig. 2-5), **(Crawford *et al*., 2005)**.

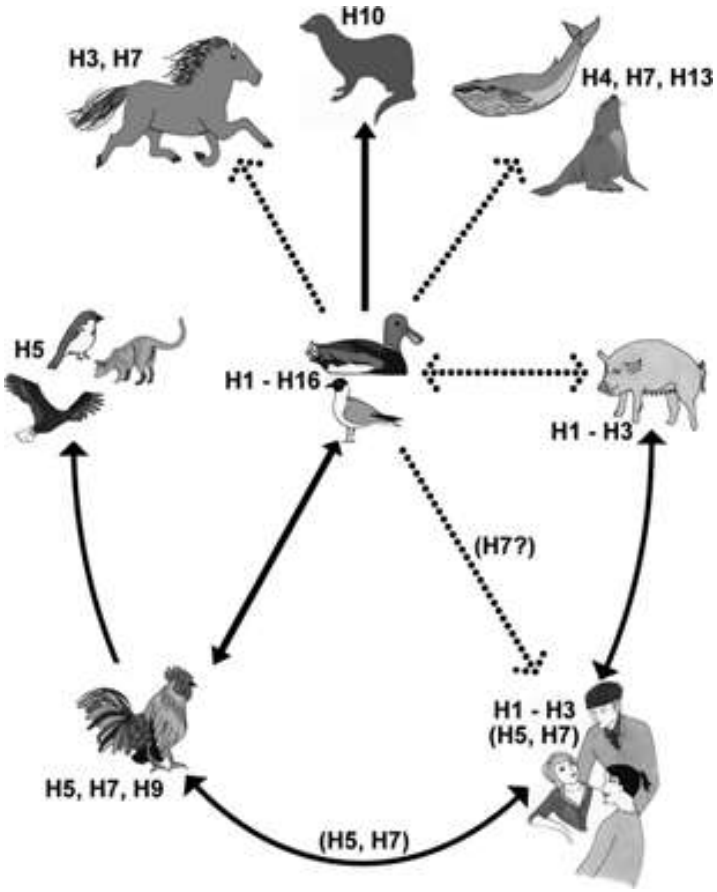

Fig. 2-5 Reservoir of influenza A viruses. The working hypothesis is that wild aquatic birds are the primordial reservoir of all influenza viruses for avian and mammalian species. Transmission of influenza virus has been demonstrated between pigs and humans (solid lines). There is extensive evidence for transmission between wild ducks and other species, and the five different host groups are based on phylogenetic analysis of the NPs of a large number of different influenza viruses (Reperant *et al*., 2009).

2.4.4 Survival of the virus

The equine influenza virus has a lipid envelope and does not remain infectious for long outside the horse. It is inactivated by exposure to ultraviolet light for 30 minutes, by heating at 50 °C for 30 minutes, and by exposure to sunlight for 15 minutes at 15 °C. It can, however, persist in water or soil under dark storage for hours. It does not survive long in high humidity or when exposed to direct sunlight for a lengthy period. In 35–45 percent humidity and at a temperature of 28°C the virus has been shown to survive on hard, non-porous surfaces such as stainless steel and plastic for 28 to 48 hours. In the same conditions it has also been shown to survive for less than eight to 12 hours on cloth and paper. Further, studies have shown that the virus can be transferred from stainless steel surfaces to hands and from paper tissues to hands. The virus can be quickly inactivated by a variety of disinfectants and chemicals. Soaps and detergents are effective because of the lipidity of the virus's envelope. The Australian Veterinary Emergency Plan (AUSVETPLAN) decontamination manual lists a range of substances that can be used; among them are soaps and detergents, oxidizing agents (including Virkon™), alkalis, acids and aldehydes **(Callinan, 2008).**

2.4.5 Pathogenesis of the virus

Equine influenza is spread via respiratory route. The virus is inhaled and infects the upper and lower respiratory tract of a susceptible horse. In order to spread the virus a horse must be shedding. The characteristic harsh cough is an effective method of transmitting the virus for up to 35 metres around the affected horse. There is also evidence that the virus can travel a considerable distance by wind. In the South African outbreak of 1986 it was claimed, anecdotally, that the

virus was carried up to 8 kilometers. In fully susceptible groups of horses infection can spread rapidly within the group and between different groups. The latter can occur as a result of the movement of recently infected horses to and from race meetings, studs, agricultural shows, pony clubs, horse sales, and anywhere else that horses mingle. Contamination can also occur if the virus is present on surfaces in horse transport vehicles or on the equipment, clothing or person of grooms, veterinary surgeons, trainers, furriers and other people who have close contact with horses. Such contamination depends on the survival of the virus on skin, fabrics and equipment and in or on vehicles. Contaminated vehicles represent a major method of spread unless subjected to careful cleaning and disinfection. They were blamed for the rapid spread of the virus in South Africa in 1986 and 2003. Vehicles often carry horses over long distances in an environment conducive to persistence of the virus**. (Callinan, 2008)**.

2.4.6 The clinical signs of equine influenza

The three most common signs of equine influenza are a deep, dry, hacking cough, onset of pyrexia (an elevated temperature, between 39°C and 41°C) and a watery nasal discharge that can later become mucopurulent. The period of pyrexia commonly occurs four to five days after infection; the coughing can persist for one to three weeks. The mucopurulent nasal discharge is a result of a secondary bacterial infection of the affected respiratory epithelium. Other signs of the disease are depression, loss of appetite, labored breathing, and muscle pain and stiffness **(AUSVETPLAN Edition 3, 2007)**.

Vaccination makes it less likely that horses will develop the disease. If they do, the clinical signs are less severe than they are in an unvaccinated horse. The

extent to which it does so depends on the efficacy of the vaccine, which is in part a function of the extent of antigenic drift that has occurred between the virus strains the vaccine contains and the challenge virus. In vaccinated horses the clinical signs just described are variable and can be difficult to discern. In some cases there might be little or no coughing or pyrexia and sub-clinical infection only—that is, infection with no clinical signs at all **(Callinan, 2008)**.

With time, the clinical signs of equine influenza become fairly easy to recognize. In the very early stages, however, and before testing, especially with horses that have travelled long distances, an impression can be gained that the illness is travel sickness, which can produce similar but less overt signs. Among other infectious and non infectious diseases affecting the upper and lower respiratory tract of horses and causing coughing, with or without fever, are not only travel sickness (bacterial bronchopneumonia or pleuropneumonia) but also equine viral arteritis, equine rhinovirus or adenovirus infection, and strangles. The similarities can cause confusion in clinical diagnosis. The main differentiating features between those diseases and equine influenza are the latter's rapid spread in unvaccinated horses, the high morbidity rate and the prominence of the deep, hacking cough **(Gagnon *et al*, 2007)**.

2.4.7 Long-term effects on horses

Recovery from equine influenza is usually uncomplicated, although coughing can, as noted, persist for up to three weeks. Mortality levels are low, but deaths have been recorded in foals and in older horses debilitated by other disease. Death in adult horses is usually a consequence of secondary bacterial infection leading to pneumonia or pleuropneumonia**,** The severity of the illness depends very much on

the immune status of the infected horse and the virulence of the virus strain. The virus does not persist in the recovered horse, and there is no evidence of any long-term carrier state after the infective period has ended **(Callinan, 2008)**.

2.4.8 Incubation and virus excretion

For detection of the illness it is important to keep in mind that there are three distinct periods—the incubation period, the latent period and the infectious period.

(a) The incubation period is the time between infection and the appearance of abnormal clinical signs. The (Office International of Epizootics) OIE Terrestrial Animal Health Code 2006 gives a maximum incubation period of five days **(OIE, Terrestrial Animal Health, 2008)**. In susceptible horse populations during severe epidemics an incubation period of one to two days has been observed. The incubation period is inversely proportional to the magnitude of the dose of the virus, which explains why the period tends to be shortest during the peak of an epidemic, when many infected horses are shedding large amounts of the virus in nasal discharge or aerosolized droplets. Longer incubation periods are associated with the infective virus requiring several rounds of replication within the horse before causing sufficient pathology for clinical signs to become apparent.

(b) The latent period is the time between infection and the start of shedding of the virus. Studies have estimated this period to be between one and four days, with a most likely period of two days. An infected horse can even start shedding before it shows clinical signs of the disease **(Park *et al.*, 2004)**.

(c) The infectious period is the period during which infected horses shed the virus and are infectious for other animals. The OIE Terrestrial Animal Code 2006 cites a maximum infectious period of 14 days **(OIE, Terrestrial Animal Health, 2008)**. however, that in susceptible unvaccinated horses shedding can persist for between seven and 10 days. Most shedding occurs in the early stages of the clinical disease, when coughing is most pronounced. Shedding can occur in partially immune horses showing no or only mild clinical signs ended **(Callinan, 2008)**.

2.4.9 Diagnostic techniques

Laboratory diagnosis of influenza virus infections is based on virus isolation from horses with acute respiratory illness, or on the demonstration of a serological response to infection. Ideally, both methods are used. Infection may also be demonstrated by detection of viral antigen in respiratory secretions using an enzyme-linked immunosorbent assay ELISA or viral genome using polymerase chain reaction PCR assays. All influenza viruses are highly contagious for susceptible hosts, including embryonated hens' eggs and cell cultures. careful handling of infected eggs or cultures Is needed to avoid accidental cross-contamination **(United States Department of Health and Human Services, 1982)**. Standard strains should not be propagated in the diagnostic laboratory, at least never at the same time or in the same place where diagnostic samples are being processed. All working areas must be efficiently disinfected before and after virus manipulations, which should preferably be conducted within biohazard containment.

It is important to obtain samples as soon as possible after the onset of clinical signs, preferably within 3–5 days. These samples include nasopharyngeal swabs and nasal or tracheal washings, the latter taken by endoscopy. Swabs may consist of absorbent cotton wool sponge/gauze on wire, and should be long enough to be passed via the ventral meatus into the nasopharynx. Swabs should be transferred to a tube containing transport medium immediately after use. This medium consists of phosphate buffered saline PBS containing 40% glycerol, or PBS containing 2% tryptose phosphate broth, 2% antibiotic solution (penicillin [10,000 units], streptomycin [10,000 units] in sterile distilled water [100 ml]), and 2% fungizone (250 mg/ml stock). If the samples are to be inoculated within 1–2 days they may be held at 4°C, but, if kept for longer, they should be stored at –70°C or below. Preferably, samples should also be transported on ice.

Only one sample is processed at a time. The liquid is expelled from the swab by squeezing with forceps, which is then disposed of suitably. Further antibiotics may be added if samples appear to be heavily contaminated with bacteria. The remainder of the fluid is stored at –70°C. Samples treated with antibiotics are allowed to stand on ice for 30–60 minutes and are then centrifuged at 1500 ***g*** for 15 minutes to remove bacteria and debris; the supernatant fluids are used for inoculation. Filtration of samples is not advised as influenza virus may adsorb on to the filter and be lost from the sample.

2.4.9.1 Identification of the agent

Isolation of infectious virus may be carried out in embryonated hens' eggs or cell cultures and EI nucleic acid can be identified by PCR. Traditionally, eggs have been preferred for isolation of equine influenza. Comparison of H3N8 viruses isolated in eggs and Madin–Darby canine kidney MDCK cells indicated that MDCK cells are capable of selecting variant viruses that are not representative of the predominant virus in clinical specimens **(Ilobi *et al*., 1994)**.

However, in recent years some viruses have been successfully isolated in MDCK cells but not in eggs and selection of variants has occurred as a result of culture in eggs **(Oxburgh and Klingborn, 1999)**, therefore isolation should be attempted using both substrates.

PCR techniques have been described for the identification of equine influenza virus from clinical specimens and for molecular epidemiology **(Donofrio *et al*., 1994; Oxburgh and Hagstrom, 1999, and Lai *et al*., 2001)**.

In situations where laboratory facilities for virus isolation are unavailable, influenza virus antigen in nasal secretions may be detected directly by an antigen-capture ELISA for the H3N8 virus using a monoclonal antibody MAb against the nucleoprotein **(Cook *et al*., 1988, and Livesay *et al*., 1993)**. Commercial self-contained kits for detecting human influenza are available and have been shown to detect equine influenza antigen **(Chambers *et al*., 1994)**.

This approach provides a rapid result on which management decisions may be based. It should not be used in preference to virus isolation, as it is essential that new viruses be isolated and sent to reference laboratories for characterisation as

part of the surveillance programme to monitor antigenic drift and emergence of new viruses and to provide isolates for inclusion in updated vaccines. Positive ELISA results are useful in the selection of samples if resources are limited or for the selection of specimens to be sent to a reference laboratory for virus isolation attempts.

(a) Virus isolation in embryonated hens' eggs

Fertile eggs are set in a humid incubator at 37–38°C and turned twice daily; after 10 11 days, they are examined by candling and live embryonated eggs are selected for use. The area above the air sac is cleansed with alcohol and a small hole is made through the shell. Several eggs/sample are inoculated (0.1 ml) in the amniotic cavity with no additional dilution of the sample (sample may also be diluted). The syringe is withdrawn approximately 1 cm and a further 0.1 ml is inoculated into the allantoic cavity. Alternatively, the sample may be inoculated into the allantoic cavity alone through a second hole drilled just below the line of the air sac. The hole(s) is/are sealed with wax or Sellotape, and the eggs are incubated at 34–35°C for 3 days. The embryos that die within 24 hours following inoculation should be discarded. The eggs that contain embryos that die more that 24 hours after inoculation or contain live embryos after 3 days are examined for the presence of EI virus.

The eggs are transferred to 4°C for 4 hours or overnight to kill the embryos and to reduce bleeding at harvest. The shells are disinfected, and the amniotic and/or allantoic fluid is harvested by pipette, each harvest being kept separate. These are tested for haemagglutination HA activity by mixing in equal volumes (0.025 ml) with chicken red blood cells RBCs (0.5% [v/v] packed cells in PBS) in

V- or U bottomed microtitre plates or 0.4% guinea-pig RBCs (0.4% [v/v] packed cells in PBS) in V- or U-bottomed plates. If chicken RBCs are used, the plates may be read by tilting to 70° so that non-agglutinated cells 'stream' to the bottom of the well. Non-agglutinated guinea-pig cells appear as a button at the bottom of the well and may take longer to settle. If there is no HA activity, aliquots of each harvest are pooled and passaged into further eggs. All HA positive samples are divided into aliquots and stored at –70°C; one aliquot is titrated for HA immediately. If the HA titre is 1/16 or more, the isolate is characterized immediately. If titers are low, positive samples should be passaged. Care should be taken to avoid generation of defective interfering particles by prediluting the inoculum 1/10, 1/100, 1/1,000. Positive samples arising from the highest dilution should be selected as stocks for storage. It may be necessary to undertake as many as five passages to isolate the virus, particularly from vaccinated horses. If virus has not been recovered by the fifth passage, further passages are unlikely to be successful.

(b) Virus isolation in cell cultures

Cultures of the MDCK cell line (MDCK, ATCC CCL34) may be used to isolate equine influenza viruses. The cells are grown to confluence in tubes and then infected in triplicate with 0.25–0.5 ml of each sample, processed as described above. Prior to inoculation, the cell monolayer is washed at least once with tissue culture medium containing trypsin (2 μg/ml) without serum. The cultures are maintained with serum-free medium containing 0.5–2 μg/ml trypsin (treated with TPCK [L-1-tosylamine-2-phenylethyl chloromethyl ketone] to remove chymotrypsin, available pretreated, e.g. from Sigma), and examined daily for evidence of cytopathic effects CPE. If positive, or after 7 days in any case, the

supernatant fluids are tested for HA. Fluids with titers of ≥1/16 are characterized immediately. Negative fluids and those with titers <1/16 are repassaged up to five passages. Alternatively, the cells are screened for evidence of haemadsorption HAD. This procedure detects expression of viral antigens at the cell surface. The medium is removed from the cultures and the tubes are washed with PBS. One or two drops of a 50% suspension of chicken or guinea-pig RBCs are added, the tubes are rotated carefully, and kept at room temperature (23°C ±2°C) for 30 minutes. Unbound RBCs are washed off with PBS, and the cultures are examined microscopically for evidence of HAD.

(c) Haemagglutinin typing

The HA subtype of new isolates of equine influenza viruses is best determined by haemagglutination inhibition using H7N7 and H3N8 specific antisera. Isolates may first be treated with Tween 80/ether, which destroys viral infectivity and reduces the risk of cross-contamination. In the case of H3N8 viruses particularly, this treatment enhances the HA activity **(Gupta *et al*, 1993)**. However, treatment with Tween 80/ether may increase the variability of the results obtained. Standard antigens must be titrated in parallel with tests to identify viruses and should include H7N7 strains (e.g. A/eq/Prague/56, A/eq/Newmarket/77) and H3N8 strains (e.g. A/eq/Newmarket/2/93, and A/eq/Kentucky/94). Virus strains may be obtained from OIE Reference Laboratories **(OIE, Terrestrial Animal Health, 2008)**. Additionally, it will be useful to include recent isolates from the same geographical area should be included if available. The standard antigens need to be treated with Tween 80/ether to avoid cross-contamination. Test antigens and standard antigens are always backtitrated to confirm their antigen content. New

isolates of equine influenza viruses may be further characterised by HI using strain-specific antisera. The species in which antibodies are raised will influence the cross-reactivity of the antiserum, with ferrets providing the most strain-specific antibody **(Mumford and Wood, 1992)**.

All isolates should be sent immediately to an International Reference Laboratory designated by OIE or the World Health Organization WHO for inclusion in the strain surveillance programme to monitor antigenic drift and emergence of new viruses.

(d) Neuraminidase typing

Typing of neuraminidase requires specific antisera and no routine technique is available. Typing can be done using specific PCR primers (**WHO, 2002)**.

(e) Polymerase chain reaction

PCR assays are increasingly used for the detection of equine influenza genome in nasal secretions. Realtime Light Cycler reverse transcription PCR technology has been shown to be more sensitive for the detection of positive samples than virus culture in eggs or detection of nucleoprotein using ELISA technology **(Quinlivan *et al*., 2005)**. Although genetic sequence of isolates can also be derived from PCR assays it remains essential to isolate infectious virus in order to examine antigenic properties of new isolates and antigenic drift.

2.4.9.2 Serological tests

Infections are detected by performing serological tests on paired sera to show a rise in antibody. These tests should be carried out whether virus isolation has been

attempted or not. Two simple methods exist, HI and single radial haemolysis SRH, each equally efficient and widely used. The complement fixation CF test can also be applied, but is not in general use. Both of the paired serum samples should be tested together at the same time to minimise variability (**WHO, 2002)**.

(a) Haemagglutination inhibition test

The antigen is first treated with Tween 80/ether in order to increase the sensitivity of the test, particularly for H3N8 viruses. The test is best done in microtitre plates using the appropriate dilution equipment. A macrotest may be used, for which antigen is diluted to a final HA titre of 1/8 per well and the volumes for PBS, sera and antigen are 0.5 ml. Sera are pretreated to remove nonspecific haemagglutinins, and inactivated at 56°C for 30 minutes.

Pretreatments include the use of one of the following: (a) kaolin and RBCs absorption, (b) potassium periodate, or (c) *Vibrio cholerae* receptor-destroying enzyme. Potassium periodate or *V. cholerae* receptor-destroying enzyme is the treatment of choice. The treated sera are diluted in PBS, a standard dose of antigen is added (HA titre of 1/4 per well for microtitration assay), and these are kept at room temperature (23°C ±2°C) for 30 minutes. After gentle mixing, RBCs are added and the test is read 30 minutes later.

The HI titres are read as the highest dilution of serum giving complete inhibition of agglutination. Either chicken RBCs (1% [v/v] packed cells) in V-bottomed microtitre plates or guinea-pig RBCs (0.5% [v/v] packed cells) in V- or U-bottomed plates may be used. If chicken RBCs are used, the plates may read by tilting to 70° so that non-agglutinated cells ‘stream’ to the bottom of the well. Nonagglutinated guinea-pig cells appear as a ‘button’ in the bottom of the well and

may take longer to settle. Titre increases of fourfold or more between paired sera indicate recent infection **(United States Department of Health and Human Services, 1982).**

(b) Single radial haemolysis

In this test, viral antigens are coupled to fixed RBCs that are suspended in agarose containing guinea-pig complement C'. Wells are punched in the agarose and filled with test sera. Influenza antibodies and C' lyse the antigen-coated RBCs, resulting in a clear, haemolytic zone around the well; the size of this zone is directly proportional to the level of strain-specific antibody in the serum sample **(Schild and Oxford, 1975, Plateau and Cruciere, 1983 and Morley *et al*., 1995)**.

Special immunodiffusion plates (MP Biomedical) may be used for the assay, but simple Petri dishes are also suitable. Sheep RBCs collected into Alsever's solution are washed three times. The C' can be obtained commercially, or normal guinea-pig serum can be used. The antigens are allantoic fluids or purified preparations; the strains used are the same as for the HI tests. The viruses are coupled to RBCs by potassium periodate or by chromic chloride. The coupled antigen/RBCs preparations are mixed with C', together with a 1% solution of agarose (low melting grade) in PBS. Care is required to ensure that the temperature is not allowed to rise above 42°C at any time. The mixture is poured into plates and left to set. Wells of 3 mm in diameter and 12 mm apart are punched in the solidified agarose, at least 6 mm from the edge of the plates. Such plates may be stored at 4°C for 12 weeks. Plates are prepared for each antigen. Sera are inactivated at 56°C for 30 minutes, but no further treatment is necessary. Paired sera should be assayed on the same plate. As a minimum, a subtype-specific antiserum should be included as

a control serum in one well on each plate. All sera are tested in a control plate containing all components except virus to check for nonspecific lysis. Alternatively, an unrelated virus, such as A/PR/8/34 H1N1, may be used in the control plate. Sera that show haemolytic activity for sheep RBCs must be pre-absorbed with sheep RBCs. Zones of lysis should be clear and not hazy or translucent. All clear zones should be measured and the area of haemolysis calculated **(OIE, Terrestrial Manual 2008)**.

2.5 Vaccination

The principal aim of influenza vaccination is to reduce clinical signs of the disease, with subsequent improved animal welfare leading to a shortened convalescent period and reducing secondary infections. Reduced shedding of virus has important implications for the spread of infection and is certainly the other major target that should be achieved by vaccination. Vaccination should also provide long-term immunity, an efficient memory response and cross-protection against influenza viruses of different strains. It has been estimated that 70% of a given population of horses needs to be fully vaccinated to prevent epidemics of influenza **(Baker, 1986)**.

In assessing vaccine efficacy in horses, clinical protection against equine influenza is defined by the absence of pyrexia, and other clinical signs induced by infection, such as nasal discharge and cough. Virological protection is defined by the absence of virus in mucosal secretions as detected by titration of virus in nasal swab extracts. ‘Seroconversion’ is defined as a significant increase of antibody.

Vaccination schedules can differ according to a country's regulations, the type of vaccine and the vaccine manufacturer's recommendations. However, a standard schedule for equine influenza vaccination requires that primarily vaccinated horses have to receive a second vaccination within 3 months. Generally, booster immunisations must be administrated within 6 months of the second vaccination and at least annually thereafter, but in some cases, boosters are given more frequently **(EquiFluNet. Global surveillance network for equine influenza, Newton *et al*, 2005)**.

Equine influenza vaccines were first developed in the 1960s in response to outbreaks in the United States in that decade. Historically, vaccination against H7N7 strains was quite successful in controlling the virus. Since the emergence of the H3N8 strains the level of protection has varied as a result of the higher rate of antigenic drift. Immunity from equine influenza is short-lived, both after vaccination and after natural infection **(OIE, Terrestrial Manual 2008**, **Callinan, 2008)**.

The effectiveness of a vaccine is determined by the level of detectable antibody produced in the exposed horse and the extent of antigenic drift that has occurred between the challenge virus and the strains of the virus in the vaccine. The level of detectable antibody produced is associated with the vaccine's potency and the time that has elapsed since the last vaccination. Until recently, equine influenza vaccines consisted of killed or inactivated whole viruses or their sub-units, with or without an adjuvant (which is used to stimulate levels of antibody).

There have been several studies of the efficacy of various commercial vaccines against different virus strains, particularly after it was demonstrated that

the H3N8 sub-type had diverged into two distinct lineages. The 2003 outbreak in South Africa and the 2007 Australian outbreak were caused by viruses in the Florida sub-lineage of the American lineage. Challenge trials have found that some currently available inactivated vaccines may offer short-term protection against these viruses.

The Animal Health Trust Laboratory is coordinating a surveillance program by the OIE and WHO reference laboratories with the aim of obtaining information on suitable vaccine strains. The surveillance panel has recommended that the H7N7 sub-type be omitted from current vaccines because no reports of infection with this sub-type have been substantiated in the past 20 years. The panel has also recommended that vaccines include representatives of both the American-like and the European-like lineages.

Notwithstanding these recommendations, many commercially available vaccines still contain H7N7 virus strains and less-than-optimal representatives of the currently circulating H3N8 viruses **(OIE, Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2004)**.

The fact that there is no perfect vaccine makes the specification of particular vaccines difficult. It does not, however, stand in the way of a requirement that vaccines containing H7N7 virus strains or that are otherwise considered ineffective be avoided; nor does it justify an abstention from continually reviewing the available catalogue and recommending the best vaccine at the time **(Callinan, 2008)**.

Current vaccination strategies can be divided into the administration of either ‘dead’ or ‘live’ vaccines. ‘Dead’ vaccines include killed whole virus, subunit

proteins and DNA vaccination. ‘Live’ vaccines include attenuated virus or living virus-based vector vaccines.

2.5.1 Dead vaccines

Since the introduction of EIV vaccines in the 1960s, the majority of equine influenza vaccines commercially available contained inactivated whole virus or subunits. The main advantages of these vaccines are the absence of pathogenicity, virus replication and subsequent spread between hosts. For the preparation of these vaccines, EIV has been traditionally grown in embryonated hens’ eggs. To reduce the subsequent reactogenicity that may occur to repeated immunisation with egg protein, methods of tissue culture have been developed.

2.5.2 Inactivated whole influenza virus vaccines

Protection from influenza disease conferred by conventional inactivated vaccines is strongly associated with the levels of circulating antibodies against HA, provided that the vaccine strain and the challenge infection strain are genetically and antigenically similar. It should be noted that the severity of disease could vary depending on the method of infection (e.g. intranasal instillation versus nebulised aerosol) and the titre of virus used for challenge infection, and these factors could influence the level of SRH antibody required for protection. However, it is clear that high levels of SRH antibody immediately prior to exposure to influenza virus play an important role in protection **(Paillot *et al*, 2006)**.

2.5.3 DNA vaccines

Administration of DNA plasmids offers a different form of vaccination and efficient protection against influenza infection has been demonstrated in several species (e.g. mice, ferrets, chicken) **(Fynan *et al*, 1993 and Webster *et al*, 1994)**.

DNA vaccination results in the in vivo expression of antigenic proteins, leading to the stimulation of both humoral and cellular immune responses. This approach is therefore advantageous compared with inactivated whole virus or sub-unit vaccines. Technically, plasmid DNA is inexpensive to produce, has good stability and can be lyophilised for long-term storage.

2.5.4 Vaccine potency

Currently, the principal markers for resistance to and recovery from influenza virus infection are circulating antibodies specific for the HA and NA glycoproteins **(Askonas and Lin, 1982)**. These glycoproteins are the principle determinants for cell entry in infection HA and for exit from the cell after virus replication NA. Progress in assessing the protective efficacy of early vaccines was hampered by a lack of reliable methods to measure the HA content of vaccines and the host's antibody response to the HA. Additionally, there was no reproducible challenge method in horses for assessing the protection provided by vaccination. The HA content of vaccines was measured in chick cell agglutination CCA units and antibody responses to the HA were measured by the haemagglutination inhibition HI test. In some instances these methods are both still used. Early attempts to analyse the relationship between vaccine-induced antibody and protection against

infection were confused by technical problems, and HI titres ranging from 8 to 128 were quoted as being protective **(Thomson *et al*, 1977)**.

The historical lack of standardisation of vaccines from different sources, and the undemanding standards of some licensing authorities, has resulted in the use of products with inadequate potency in terms of ability to stimulate antibody to the HA.

2.5.5 Natural immunity and live vaccines

Immunity provided by inactivated influenza virus vaccines, is dependent on high levels of circulating antibody to HA and, in the absence of such antibody, vaccinated horses are susceptible to infection. In contrast, infection with influenza induces longterm immunity independent of circulating antibody against HA. For example, ponies with low or undetectable anti-HA antibodies were clinically and virologically protected from challenge infection more than one year after natural infection **(Hannant *et al*, 1988)**.

Equine influenza virus infection has been demonstrated to generate virus-specific mucosal IgA and serum IgGa and IgGb responses, whereas an inactivated virus vaccine induced only a serum IgG (T) response **(Nelson *et al*, 1998)**.

2.5.6 Vacine strain selection

Surveillance of antigenic drift is a cornerstone of influenza control programmes based on vaccination. As with other RNA viruses, influenza virus replication is highly error-prone, therefore newly synthesised viral genes have a high frequency of mutation. Many of these mutations are either inconsequential or detrimental to the virus, but mutations affecting the antigenic sites of the HA and NA can lead to

the virus not being recognisable by pre-existing antibodies generated by infection or vaccination with an earlier strain, a process known as “antigenic drift”. The formulation of human influenza vaccines is reviewed on an annual basis and in most years is changed to reflect the virus strains most representative of those in worldwide circulation.

Historically, antigenic drift in equine H3N8 viruses has been examined in HI tests employing post infection or post vaccination sera prepared in a number of different species. Conclusions about the antigenic relatedness of equine H3N8 viruses and the significance of observed differences with respect to the immunity induced have varied. For example, **(Hinshaw *et al,* 1983)** concluded than the majority of viruses isolated between 1979 and 1981 were substantially different from the prototype virus, Miami/ 63 included in the vaccine when compared using post infection ferret sera in HI assays, and that representatives of the new variant should be included in the vaccines. On the other hand, **(Burrows and Denyer, 1982)** concluded that the minor antigenic drift that they detected in viruses isolated between 1963 and 1979 did not justify a change in vaccine strains because post vaccination sera from horses immunised with Miami/63 virus were highly cross-reactive in HI tests with viruses from 1979. This conclusion did not take into account the findings of (**Haaheim and Schild, 1980)** that strain-specific antibody is more effective than cross-reactive antibody in conferring protection.

During the 1989 outbreak of influenza in the UK, only horses with very high levels of vaccine-induced antibody were protected against infection, raising the possibility that there had been significant antigenic changes in the 1989 isolate that prevented its neutralization by antibody stimulated by vaccines containing

Miami/63, Fontainebleau/79 or Kentucky/81. Sequencing of the HA1 gene and antigenic analysis using monoclonal antibodies suggested that there were significant differences between a representative 1989 strain and the vaccine strains in current use at the time **(Binns *et al*, 1993)**.

Although all vaccines provided clinical protection, vaccine efficacy in terms of ability to eliminate virus excretion correlated directly with the degree of antigenic relatedness between vaccine and challenge strain. Following a meeting of OIE and WHO experts on newly emerging strains of equine influenza, it was recommended that equine influenza vaccines be updated to include a 1989 isolate, and that efforts be made to increase surveillance and virus characterization **(Mumford and Wood, 1993)**.

Chapter III

Materials and Methods

3.1 Materials

3.1.1 Materials for cell culture and viral propagation

1. Cells: Median Derby Canine Kidney MDCK cell line was kindly provided by Dr. Essam Azhar, King Fahad Research Centre level 3 as a confluent sheet in 75 cm^2 plastic tissue culture flask.

2. Fetal Bovine Serum FBS was purchased from Biowest, Biowest®, France Cat. No. A1111-L.

3. Antibiotic-Antimycotic Mixture was purchased from GIBCO-BRL; New York, USA) Cat. No. 15140-023.

4. Dulbecco's modified Eagle's minimal essential medium DMEM was purchased from BioWhittaker; Walkersville; Md. Cat. No. 12-614F.

5. Bicarbonate: $NaHCO_3$ was purchased from Analar, England Cat. No. 205-633-8.

6. Hydrochloric Acid HCl was purchased from (Analar, England) Cat. No. H9892.

7. Ethylene diamine tetra acetic acid: EDTA was purchased from BDH Chemicals Ltd, England .Cat. No. 28021.

8. **H7N7 control positive** We got from the National Research Center in Cairo by Professor Mohamed Ahmed Ali.

3.1.2 Materials for virus-RNA extraction and RT-PCR

1. **RNA extraction reagents:** Viral RNA extraction reagent kit was purchased from Qiagen (QIAamp; Qiagen, Hilden, Germany) Cat No.52904.

2. **Diethylpyrocarbonate DEPC** was purchased from Sigma; Deisenhofen, Germany Cat No. 1609-47-8**.**

3. **Reverse transcriptase** M-MLV Cat No. M1701, ***Taq* polymerase** Cat No. M3001 **and deoxynucleotide triphosphate** Cat. No. U1330 were obtained from Promega (Madison, USA).

3.1.3 Oligonucleotide primers

For H7, the following forward F and reverse R primers were designed based on previously published sequence using the Lasergene sequence analysis software (DNASTAR Inc., Madison, USA) to amplify partial sequence of a 244 bp segment, 241 bp (inner nucleoprotein primers), and 244 bp (matrix primers) were visualized on 1.2 or 1% agarose gels stained with ethidium bromide.

H7-F: 5'- ATGAGYCTTYTAACCGAG GTC GAAACG-3'

H7-R: 5'- TG GACAAAN CG TCTACGCTGCAG -3' **(WHO, 2009).**

N7-F: 5'- AGCAAAAGCAGGGTAGATAA - 3'

N7-R: 5'- TCCTTGCATCAGAGAGCACA - 3' **(Michelle, *et al.* 2004).**

3.1.4 Reagents for agarose gel electrophoresis of PCR Product

1. **Tris base** (2-amino-2-hydroxymethyl-propane-1,3-diol) (BDH Chemicals Ltd, England) Cat. No. 27119.
2. **Glacial acetic acid** (BDH Chemicals Ltd, England) Cat. No.27013.
3. **Bromophenol blue** (Sigma, USA) Cat. No. 115-39-9.
4. **Xylene cyanol FF** (Sigma.USA) Cat. No. 2650-17-1.
5. **Sucrose** BDH Cat. No. BDH8029.
6. **Ethidium bromide EB** (BDH Chemicals Ltd, England) Cat. No. 214-984-6.
7. **DNA Marker** (SibEnzyme Ltd, Russia Cat. No. M15.
8. **Agarose** (Roche, Spain) Cat. No. 11811659400.

3.1.5 Reagents for Enzyme Linked Immunosorbent Assay (ELISA)

We use the Influenza A H7 N7 hemagglutinin HA antigen (sino biological Inc.) cat. No. 11082.

1. **Sodium bicarbonate.** AVO NCHE Cat. No. 205-633-8.
2. **Sodium carbonate.** MB Biomedical Cat. No. 191437.

3. **Tween-20** TEDIA company Cat. No. 9005-64-5.

4. **Fetal calf serum** Sigma Cat. No.N 4762-100ml**.**

5. **Citric acid anhydrous** AlfaAesar Cat. No. 6131-90-4.

6. **Sodium phosphate dibasic** MP Biomedical Cat. No. 195501.

7. ***O*-phenylenediamine** OPD; 0.04%, Sigma, Deisenhofen, Germany**.** Cat. No. P5412.

8. **Hydrogen peroxide (H_2O_2; 0.006%)** Sigma, Deisenhofen, Germany. Cat. No. 316989**.**

9. **Sulfuric acid H_2SO_4** MB Biomedical Cat. No. 7664-93-9**.**

3.1.6 Hemagglutination and Hemagglutination Inhibition test

3.1.6.1 Buffers and reagents:

1. **Red blood cells** in Alsever's solution (turkey and human type "O" RBCs).

2. **Sterille distilled H_2O**.

3. **Phosphate-buffered saline** (0.01M), PH 7.2 PBS.

4. **Physiolgical saline**, 0.85% NaCl.

3.1.7 Equipment

3.1.7.1 DNA- thermal cycler (Applied biosystem, Japan)

3.1.8 Supplies

3.1.8.1 Pipettes and pipettor.

3.1.8.2 Multichannel pipettors.

3.1.8.3 Tubes and racks.

3.1.8.4 Centrifuge tubes (graduated conical 50 ml and 15 ml).

3.1.8.5 Gauze squares.

3.1.8.6 96-well microtiter plates

Costar 3897: V-shaped for use with chicken or turkey RBCs.

Costar 3797: U-shaped for use with human type "O" or guinea pig RBCs.

3.1.8.7 50-ml tubes for antigen dilutions.

3.2 Methods

3.2.1 RT-PCR

3.2.1.1 Virus RNA Extraction

Viral RNA extraction was accomplished using Viral RNA extraction reagent kit. Manufacturer´s instructions were followed. About 140 µl of the virus sample was mixed with 560 µl AVL buffer containing carrier RNA in an RNase free tube and vortex for 15 sec and then incubated at room temperature RT for 10 min. Mixture was then centrifuged briefly and absolute ethanol (560 µl) was added and vortexed for 15 sec and then briefly centrifuged. A 630 µl of the mixture was carefully applied to the spin column attached to 2 ml collection tube and centrifuged at 6000*g* (8000 rpm) for 1 min. Tube containing the filtrate was discarded and transferred the spin column into a fresh 2 ml collection tube, closed the column to avoid cross contamination while centrifuging at 6000*g* (8000 rpm) for 1 min to get rid of the residual fluid. Repeated the above step till the whole sample mix pass through the filter. 500 µl buffer AW1 was added to the spin column and closed the cap and centrifuged 6000*g* (8000 rpm) for 1 min and discarded the tube containing the filtrate. Then Placed the spin column into a fresh 2 ml collection tube and added 500 µl buffer AW2, closed the cap and centrifuged at maximum speed for 3 min and discarded. The column was Placed in a RNase free 1.5 ml tube and 60 µl buffer AVE was added (RNA elution buffer; RT), after that incubated at RT for 1 min then centrifuged 6000*g* (8000 rpm) for 1 min. The extracted RNA was divided into 10 µl aliquots, and frozen at -80 ^{o}C till used.

3.2.1.2 Reverse transcription of extracted RNA

Reverse transcription of extracted RNA was performed for synthesis of cDNA. Following the method of **(Sguazza *et al.* 2009).** mixture of 9 µl viral RNA, 1 µl Uni12 reverse primer (200 nMol/µl) and 5 µl DEPC treated water was first heated at 70 ºC in the heating block of the DNA-thermal cycler for 5 min. Such mix was chilled on ice followed by adding 1 µl of 10 mM dNTPs, 1 µl RNase inhibitor (40U), 5 µl of 5X RT-buffer, 2 µl DEPC-water and 1 µl M-MLV-RT (20U). Mixture was returned to the heating block of the DNA-thermal cycler. The cDNA synthesis program included reverse transcription stage at 42 ºC for 90 min linked to RT-inactivation stage at 94 ºC for 10 min.

3.2.1.3 Polymerase chain reaction (PCR) of H7 and N7

According to the method of **(Martella *et al.*, 2007)** with modification, the synthesized cDNA (5 µl) was mixed with 1 µl of 10 mM of dNTPs, 1 µl of each of the F and R primers (200 nMol/µl), 10 µl of 5X Taq buffer, 2 µl of 25 mM MgCl2, 1 µl (5U) Taq DNA polymerase and 29 µl DEPC-treated water giving a final reaction volume of 50 µl in 0.2 ml Eppendorf tube. The tube was placed in the DNA-thermal cycler and applied for amplification program included the following stages: initial denaturation at 94 °C for 3 min linked to 40 cycles each consisted of 3 stages including denaturation at 94 °C for 1min, annealing for 1min (at 58 °C for H7 and at 55 °C for N7) and extension at 72 °C for 1 min. The last cycle was linked to a final extension step at 72 °C for 10 min.

3.2.1.4 Visualization of RT-PCR product

A volume of 2 µl of 6X gel loading dye was mixed with 8 µl of the PCR products. Mixture was loaded on to the 2% agarose gel. 5 µl of Diluted Marker was also loaded on the same gel. Agarose gel was placed in the electrophoresis apparatus and attached to the power supply set up as a voltage of 1-5 volt/cm and the electrophoresed for 30-45min. The resolved bands were visualized on the gel documentation system. The bands were analyzed in comparison to the 100bp DNA marker using the Lab image analyzer software version 2.7.0

3.2.2 Hemagglutination and Hemagglutination Inhibition test

3.2.2.1 Reference antisera to HA subtypes

Reference antisera to many but not all of the 15 HA subtypes have been prepared in goats or sheep. These reference antisera were prepared with isolated HA and are considered monospecific. In the cases where monospecific antisera is not yet available polyclonal antisera to the reference virus in those subtypes has been prepared. In some subtypes that contain human, swine, and equine viruses, multiple antisera are included to more accurately reflect the antigenic diversity within a subtype.

These reference sera are designed to distinguish between subtypes but are broadly cross reactive to detect as many different variants as possible within a subtypes. Reference antisera for field isolate identification are prepared in sheep by multiple intramuscular injections with purified HA or in chickens by intravenous inoculation with virus grown in embryonated

eggs. Control antigens consist of infected allantoic fluid inactivated by beta-propiolactone. The preparations are derived from either the wild type vaccine which we got from the Ministry of Agriculture and Livestock in Jeddah and the strain in the vaccine is H7N7.

3.2.2.2 Preparation of reagents and solutions

i. Phosphate-buffered saline (0.01M), pH 7.2 (PBS)

a) Prepare stock 25 times concentrated (25X) phosphate buffer containing in 100 ml: 2.74 g dibasic sodium phosphate Na2HPO4 and 0.79 g monobasic sodium phosphate monohydrate NaH2PO4 x H_2O.

b) To prepare PBS, mix and dissolve in deionized, distilled water, and q.s. to 1 liter: 40 ml of 25X phosphate buffer and 8.5 g of sodium chloride NaCl.

c) After thorough mixing, check pH = 7.2 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary.

d) Autoclave or filter to sterilize.

e) Store opened PBS, pH 7.2 at 4oC for no longer than 3 weeks.

ii. Alsever's

a) Weigh out, dissolve in distilled water, and q.s. to 1 liter:

20.5 g dextrose

8.0 g sodium citrate dihydrate ($Na_3C_6H_5O_7 \times 2H_2O$)

4.2 g sodium chloride NaCl

0.55 g citric acid $C_6H_8O_7$

b) After thorough mixing, check pH = 6.1 plus or minus 0.1. Adjust pH with 1 N NaOH or 1 N HCl, if necessary. Sterilize by filtration through a membrane filter with 0.22 μm pore size. Contamination is likely in this solution if it is not adequately sterilized.

iii. Physiological saline, 0.85% NaCl

a) Prepare a 20x stock solution by dissolving 170 g of NaCl in deionized water q.s. for 1000 ml.
b) Sterilize by autoclaving at 121 °C.
c) To prepare physiological saline, 0.85% NaCl, add 50 ml 20x stock solution to 950 ml deionized water.
d) Sterilize by autoclaving at 121 °C.
e) Store opened physiological saline at 4 °C for no longer than 3 weeks.

iv. Standardized RBCs

3.2.2.3 Procedure for HAI identification of field isolates

i. Treatment of Reference Antisera for Inactivation of Nonspecific Inhibitors

1. Reconstitute lyophilized reference antisera with sterile distilled H_2O to volume indicated on label. Store reconstituted antisera at -20 °C to -70 °C.
2. Reconstitute and store RDE (receptor destroying enzyme).

a) Reconstitute the RDE with 25 ml physiological saline, 0.85% NaCl.

b) Aliquot and store at -20 °C to -70 °C.

3. Add 3 vol of RDE to 1 vol serum (0.9 ml RDE + 0.3 ml serum).

Note: This volume is sufficient for testing 50-55 field isolates.

4. Incubate overnight in a 37 °C waterbath.

5. Heat in a 56 °C waterbath for 30 min to inactivate remaining RDE.

6. Allow antisera to cool to room temperature. Add 6 vol (1-8 ml) of physiological saline, 0.85% NaCl. The final dilution of antisera is 1:10.

ii. Identification of Nonspecific Agglutinins in Treated Sera

1. Choose the appropriate plate and add 25 µl of PBS (pH 7.2) to B through H (B1-H6) wells of each of six numbered columns.
2. Add 50 µl of PBS (pH 7.2) to the first well A6 in column number 6 for a RBC control.
3. Add 50 µl of each treated antiserum to the first well (A1 - A5) of row A.
4. Prepare serial twofold dilutions of the antisera by transferring 25 µl from the first well of number columns 1-6 to successive wells in each column. Discard the final 25 µl after row H.
5. Add 25 µl of PBS, pH 7.2 to all wells of each of the six numbered columns.
6. Add 50 µl of standardized RBCs.
7. Mix by using a mechanical vibrator or by manually agitating the plates thoroughly.
8. Incubate the plates at room temperature (22 °C to 25 °C) for the

appropriate time by checking the cell control for complete settling of RBCs. Thirty minutes is usually required for chicken or turkey RBCs. Sixty minutes is required for guinea pig or human type "O" RBCs. (Table3-1).

(Table 3-1) : Influenza Hemagglutination with Different Species of RBCs

	Chicken	**Turkey**	**Guinea Pig**	**Human Type O**
Concentration	0.5%	0.5%	0.75%	0.75%
Microtiter plate	"V"	"V"	"U"	"U"
Incubation time, 25 °C	30min	30min	1 hour	1 hour
Appearance of control cells	Button	button	Halo	Halo

iii. Adsorption of Antisera to Remove Nonspecific Agglutinins

1. To one volume of packed RBCs, add 20 vol of RDE-treated serum.
2. Mix thoroughly and incubate at 4 °C for 1 hr, mixing at intervals to resuspend cells.
3. Centrifuge at 1200 rpm for 10 min.
4. Carefully remove the adsorbed serum without disturbing the packed cells.
5. Repeat serum controls as described above.
6. Repeat adsorption with RBCs until the serum controls are negative.

iv. HA Titration of Control Antigens and Field Isolates

1. Choose V-shaped 96-well microtiter plates if using chicken or turkey. RBCs. Choose U-shaped microtiter plates if using guinea pig or human type O RBCs.

2. Add 50 µl of PBS (pH 7.2) to #2 through #12 (A2 - H12) wells of each lettered row.
3. Add 100 µl of each control antigen or field isolate to the first well (A1- F1) of the lettered rows except rows G & H.
4. Prepare an RBC control well in row H H1 by adding 100 µl of PBS.
5. Make serial twofold dilutions by transferring 50 µl from the first well of lettered rows to successive rows. Discard the final 50 µl.
6. Add 50 µl of RBC suspension to each well on the plate.
7. Mix by using a mechanical vibrator or by manually agitating the plates thoroughly.
8. Incubate the plates at room temperature (22 °C to 25 °C). Check cell control for complete settling of RBCs.
9. Record results.

v. Preparation of Standardized Antigen for the HAI Test and "Back Titration"

A "unit" of hemagglutination is not a measure of an absolute amount of virus, but is an operational unit dependent on the method used for HA titration. An HA unit is defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension.

1. Determine the volume of standardized antigen needed for the HAI test. For example, 1 ml of antigen will test 5 sera, each of which is diluted in 8 wells, with 25 µl of antigen added to each well (5 sera X 8 wells X 25 µl = 1 ml of standardized antigen). Prepare an additional 1.0 ml additional volume for "back titration" and wastage.

2. The standard for the HAI test is 4 HA units of virus/antigen added to twofold dilutions of antisera. Since we are adding 25 µl of antigen in the test, we need a virus dilution that contains 4 HA units/ 25 µl or 8 HA units/50 µl. Calculate the antigen dilution by dividing the HA titer (which is based on 50 µl) by 8 because you wish to have 8 HA units/50 µl. For example, an HA titer of 160 divided by 8 is 20. Mix 1 part of virus with 19 parts PBS to obtain the desired volume of standardized antigen (Ex: Add 0.1 ml antigen to 1.9 ml of PBS). Calculate and prepare dilution.

   Keep a record of the dilution prepared.
3. Perform a "back titration" to verify units by performing a second HA test using the standardized antigen dilution preparation. Store the diluted antigen at 4 ºC and use within the same day.
4. Record results.

vi. HAI Test for serologic diagnosis

1. Label appropriate microtiter plates.
2. Add 25 µl of PBS to wells B through H (B1 - H12) of each numbered column.
3. Add 50 µl of each treated serum (1:10) to the appropriate first well (A1 - A12) of the numbered column.
4. Prepare serial twofold dilutions of the treated sera by transferring 25 µl from the first well of the numbered columns 1-12 to successive wells. Discard the final 25 µl after row H.

5. Add 25 µl of standardized antigen to all wells (A1 - H12) in a set of treated sera.
6. Add 25 µl of PBS instead of antigen to the set of treated sera for serum controls (A1 - H12).
7. Mix the contents of the plates by shaking on a mechanical vibrator for 10 sec or by agitating the plates manually.
8. Cover the plates and incubate at room temperature (22 °C to 25 °C.) for 30-45 min.
9. Add 50 µl of standardized RBCs to all wells. Mix as before.
10. Cover the plates and allow the RBCs to settle at room temperature (22 °C to 25 °C) for the appropriate time according to RBCs being used.
11. Record the HAI titers.

3.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

3.2.3.1 Preparation of reagents and solutions

i. Bicarbonate/carbonate coating buffer (100 mM)

Antigen should be diluted in coating buffer to immobilize them to the wells

- 3.03 g Na_2CO_3,
- 6.0 g $NaHCO_3$
- 1000 ml distilled water,
- pH 9.6

ii. PBS

- 1.16 g Na_2HPO_4,
- 0.1 g KCl,
- 0.1 g K_3PO_4,
- 4.0 g NaCl (500 ml distilled water)
- pH 7.4

iii. Blocking solution

Commonly used blocking agents are 1% BSA , serum, non-fat dry milk, casein, gelatin in PBS.

iv. Wash solution

Usually PBS or Tris -buffered saline (pH 7.4) with detergent such as 0.05% (v/v) Tween20 (TBST).

v. Antibody dilution buffer

Primary and secondary antibody should be diluted in 1x blocking solution to reduce non-specific binding.

3.2.3.2 ELISA Test for serologic diagnosis

1. Dilute antigen to a final concentration of 1-20 µg/mL using PBS or Bicarbonate/carbonate coating buffer. Coat the wells of a PVC microtiter plate with the antigen by pipeting 50 µl of the antigen dilution in the top wells of the plate. Dilute down the plate as required. Seal the plate and incubate overnight at 4 °C or 2 h at room temperature.
2. Wash plate 3 times with PBS.

3. Block the remaining protein-binding sites in the coated wells by adding 200 µl blocking buffer, 5% non fat dry milk/PBS, per well. Alternative blocking reagents include blockACE or BSA.
4. Cover the plate with an adhesive plastic and incubate for at least 2 h at room temperature or, if more convenient, overnight at 4 °C.
5. Wash the plate 3 times with PBS.
6. Add 100 µl of diluted primary antibody to each well.
7. . Cover the plate with an adhesive plastic and incubate for 2 h at room temperature.
8. Wash the plate 4 times with PBS.
9. Add 100 µl of conjugated secondary antibody, diluted at the optimal concentration (according to the manufacturer) in blocking buffer immediately before use.
10. Cover the plate with an adhesive plastic and incubate for 1-2 h at room temperature.
11. . Wash the plate 5 times with PBS.
12. . Dispense 100 µl (or 50 µl) of the substrate solution per well with a multichannel pipet or a multipipet.
13. After sufficient color development (if it is necessary) add 50-100µl of stop solution to the wells
14. Record the absorbance at 450 nm on a plate reader within 30 minutes of stopping the reaction. And the cutoff = 0.245

Chapter IV

Results

According to the latest statistical recorded for the year 1433 H of the King Abdul-Aziz Center for Arabian Horses in Dirab (kaahcfao) the number of horses registered in the Kingdom of Saudi Arabia 10628 horses (www. Kaahcfao.com). A total of 507 serum samples were tested for equine influenza viruses EIV and the immune status. Samples were collected from regions of KSA (Eastern, Central, Western, Southern and Northern).

out of 507 samples there was 100 nasopharyngeal swabs were placed immediately in vials containing 5 ml of phosphate-buffered saline (PBS) with 10% glycerol and antibiotics.

1. Demographic characteristics of the studied population according to age

Serum samples according to age divided to four groups < 1 year; 1-10 years; 11-20 years and >20 years. The maximum number of study population was in age group 1-10 years (74.4%) while remaining population age groups ranged from 3-16.1% as shown in Table 4-1.

Table 4-1: Demographic of study population according to age

Age in years	Total	
	No.	%
< 1 year	33	6.5
1-10 years	377	74.4
11-20 years	82	16.1
>20 years	15	3.0
Total	507	100%

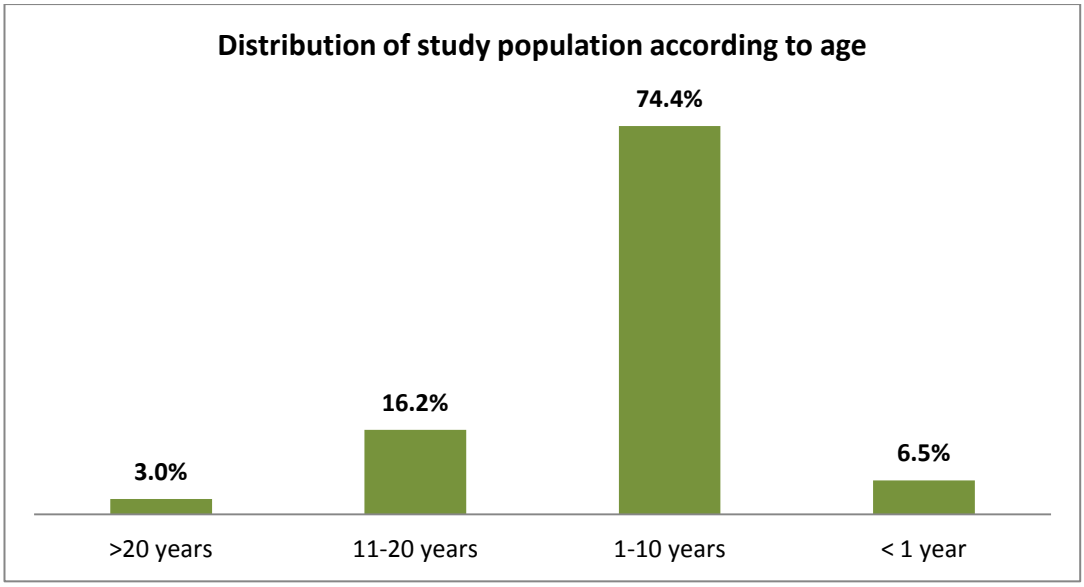

Diagram 4-1: Distribution of study population according to age

2. Distribution of study population according to age and sex

Serum samples according to sex divided to two groups males (55.6%) and female (44.4%) 282 were male horses and 225 were mare (female horse). The distribution of study population according to age and gender both horses and mare were in maximum number (76% and 72%, respectively) in age group 1-10 years followed by age 11-20 years (13% and 20% respectively) these were shown at Table 4-2.

Table 4-2: Distribution of study population according to age and gender

Age in years	Horses		Mare		Total
	No.	%	No.	%	No.
< 1 year	22	8	11	5	33
1-10 years	214	76	163	72	377
11-20 years	37	13	45	20	82
>20 years	9	3	6	3	15
Total	282	100%	225	100%	507

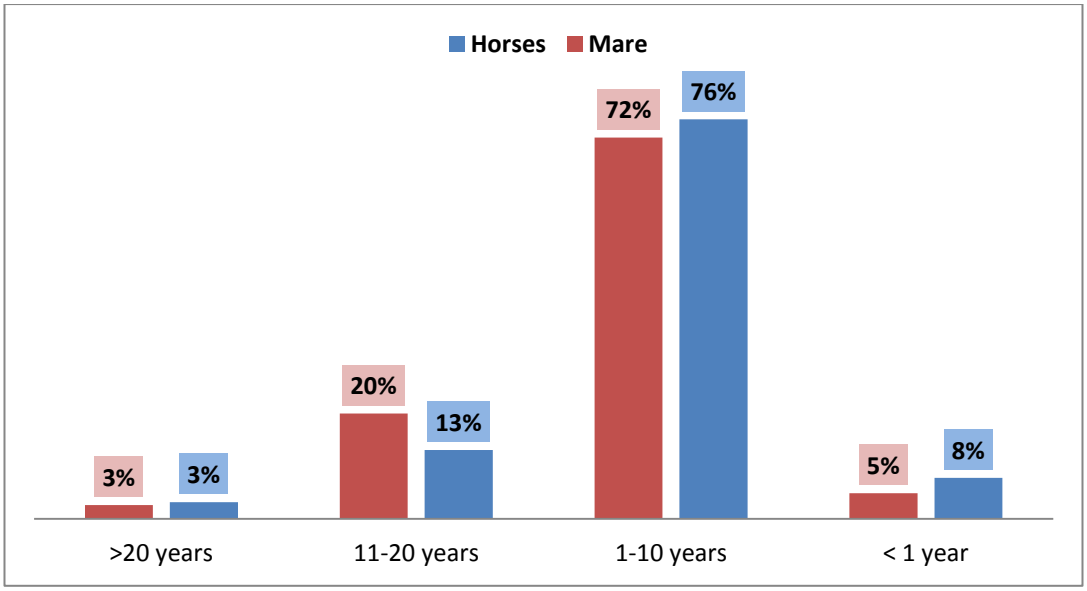

Diagram 4-2: Distribution of study population according to age and gender

3. Distribution of study population according to Regions of KSA

Serum samples according to Regions of KSA divided to five group's Eastern region, Central region, Western region, Southern region and Northern region. There were three different horses strain from five different regions of KSA. The relationship between horses strain and different regions of KSA is given in Table 4-3(a, b). The maximum number of study population was from Western region (29.6%), Eastern region (23.1%), Central and Northern region (18.5% and 18.3%) respectively.

Among three different strains, Arabic strain was more predominantly seen in Western region (35%) followed by Eastern region (25%) and Northern region (23%). Lowest percentage (3%) of Arabic strain was found in Southern region.

The Hybrid strain was also predominantly in Western region (35%). Both Eastern and Central region had hybrid strain in equal percentage (25%). Again Southern region had the strain in low number (1%). The English strain was the most common in Southern region, followed by Central region (22%), Eastern region (16%).

Table 4-3a: Relationship between horses strain and different regions of KSA

Regions of KSA	Arabic strain		Hybrid strain		English strain		Total	
	No.	%	No.	%	No.	%	No.	%
Eastern region	65	25	35	25	17	16	117	23.1
Central region	37	14	34	25	23	22	94	18.5
Western region	93	35	48	35	9	9	150	29.6
Southern region	7	3	1	1	45	43	53	10.5
Northern region	62	23	20	14	11	10	93	18.3
Total	264	100%	138	100%	105	100%	507	100%

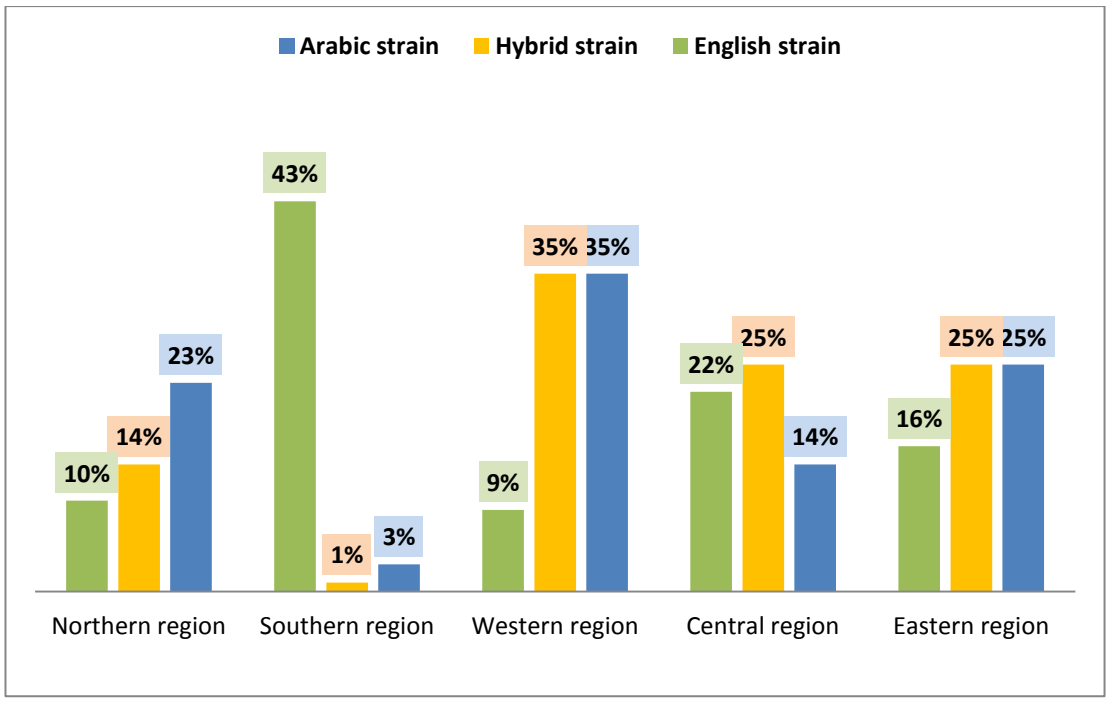

Diagram 4-3a: Relationship between horses strain and different regions of KSA

All serum samples 507 horses were the relationship between horses strain and age. The maximum number of study population was in Arabic strain, hybrid strain and English strain in horses with age 1-10 years (51%, 27% and 21%, respectively) while the minimum number in hybrid strain, English strain and Arabic strain was (13%, 19% and 69%, respectively) in age >20 years. These were shown at table 4-3b.

Table 4-3b: Relationship between horses strain and Age

Age in years	< 1 year		1-10 years		11-20 years		>20 years	
strain	No.	%	No.	%	No.	%	No.	%
Arabic strain	21	64	193	51	39	48	11	69
English strain	2	6	81	21	19	23	3	19
Hybrid strain	10	30	103	27	23	28	2	13
Total	33	100%	377	100%	81	100%	16	100%

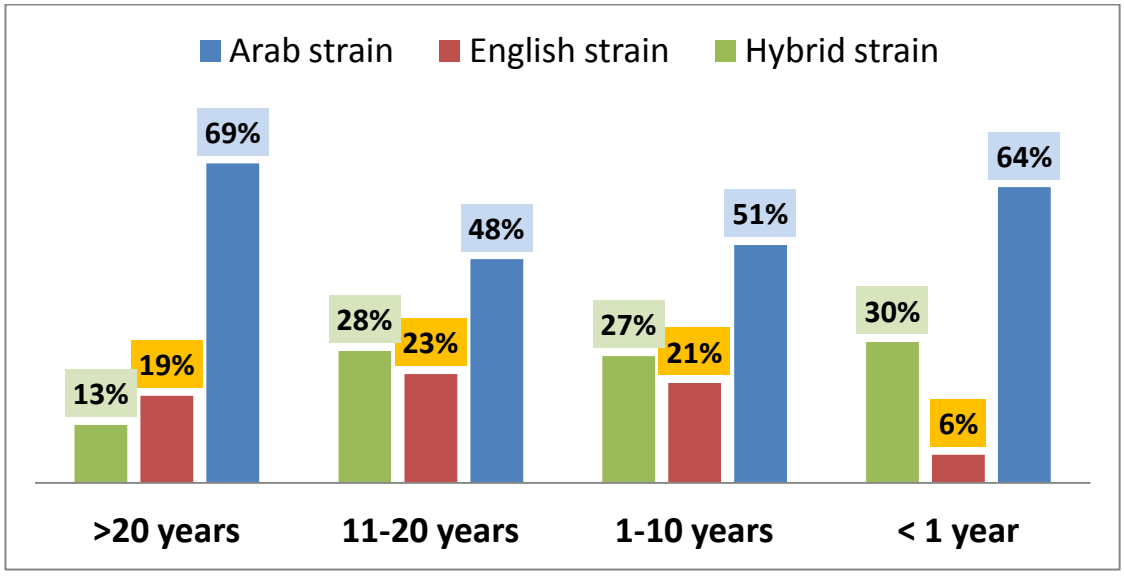

Diagram 4-3b: Relationship between horses strain and Age

4. Haemagglutination inhibition test (HI)

The HI test of equine influenza viruses in the all samples 507 Blood studded were the relationship between horses' gender and HI test (fig. 4-1). These result in demonstrate that HI titers of O+ blood are higher in comparison to the HI titers of turkey blood. These results indicate that O+ blood is sensitive to the antibodies for equine influenza present in horse serum.

i. Haemagglutination inhibition test according to gander

The 14 samples (5%) of 282 horse was positive samples (titer = 1280) in table 5a, while 30 samples (13%) of 225 mare was positive samples (titer = 1280) with turkey blood and 21 samples (9%) of 225 mare was positive samples (titer = 1280) with O+ blood. These were shown at Table 4-4(a, b).

Table 4-4a: Relationship between Horse gander and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	149	53	106	38
10	41	15	73	26
20	26	9	31	11
40	20	7	26	9
80	14	5	10	4
160	7	2	10	4
320	7	2	8	3
640	4	1	4	1
1280	14	5	14	5
Total	282	100%	282	100%

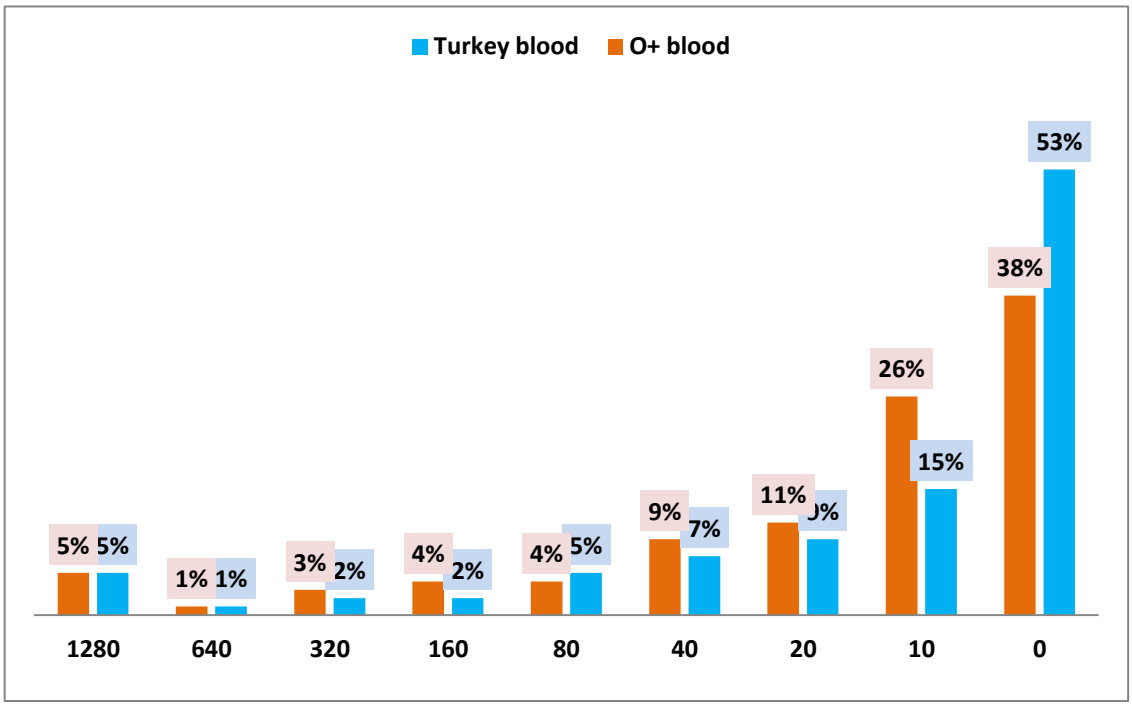

Diagram 4-4a: Relationship between Horse gander and HI test

Table 4-4b: Relationship between horses mare and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	124	55	111	49
10	21	9	29	13
20	16	7	18	8
40	13	6	21	9
80	10	4	8	4
160	6	3	11	5
320	3	1	4	2
640	2	1	2	1
1280	30	13	21	9
Total	225	100%	225	100%

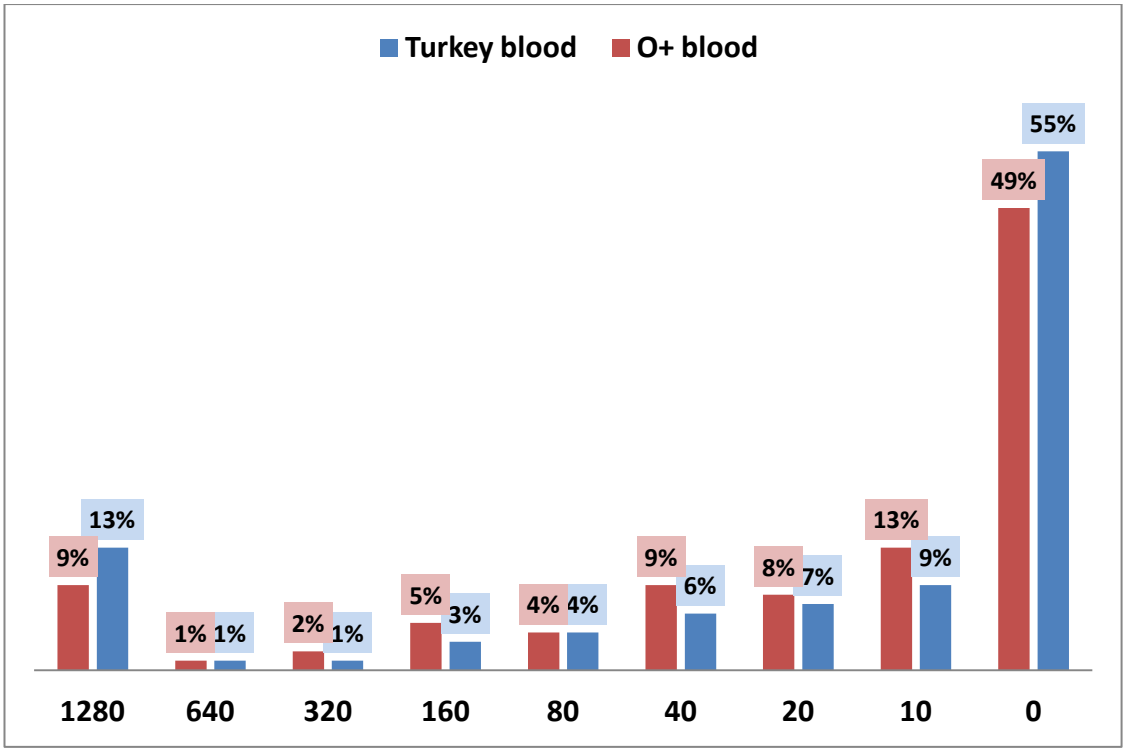

Diagram 4-4b: Relationship between horses mare and HI test

ii. Haemagglutination inhibition test according to age

The HI test of equine influenza viruses in the all samples 507 Blood studded and found the relationship between horses age and HI test as shown in Table 4-4 (c, d, e and f). In table 4-4c, there is 7 samples (21%) was positive samples (titer = 10) with turkey blood and 13 samples (39%) was positive samples (titer = 10) with O+ blood of 33 horse < 1 year.

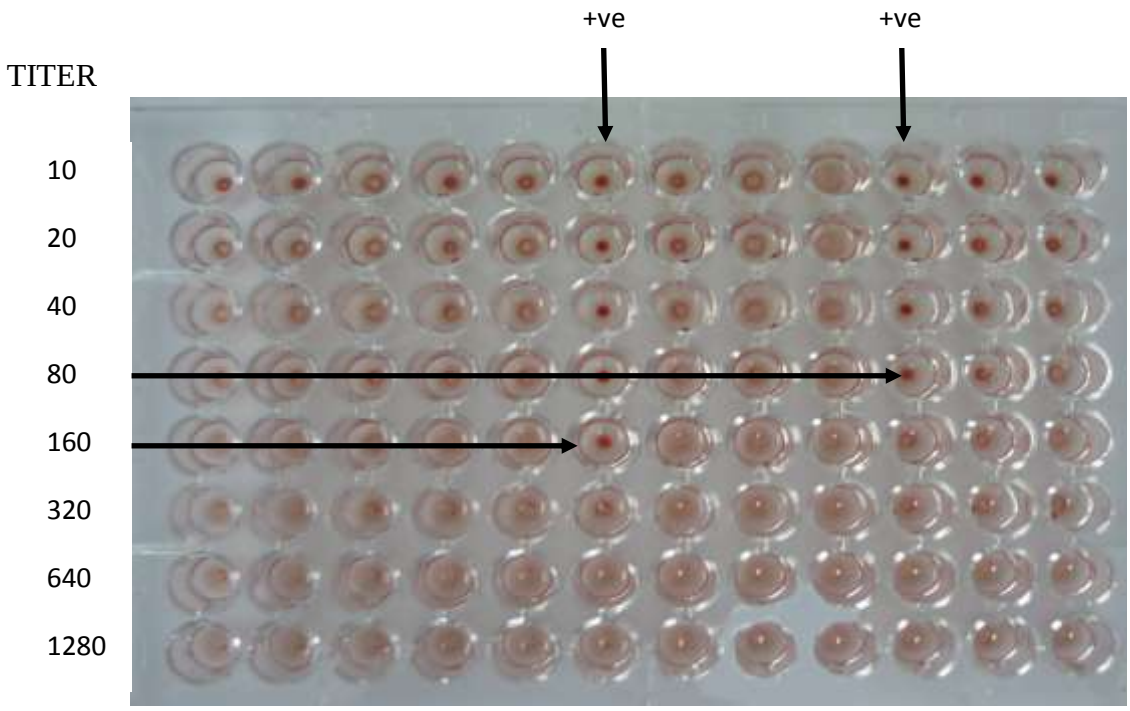

Figure 4-1: the haemagglutination–inhibition test is based on recognition of virus by a panel of reference antibodies. Influenza viruses bind to red blood cells using the haemagglutinin molecule and agglutinate them, a process which is easily seen if virus and red blood cells are mixed together in the correct proportions and plated out in a 96-well plate. By serially diluting specific antibodies and adding these to the virus it is possible to block this interaction and measure how closely the virus is related to the antisera and to previous strains. In the figure above, the top row of wells shows a weak interaction between virus and antiserum (turkey blood) (titre = 10), the bottom row shows a strong interaction (titre = 160).

Table 4-4c: Relationship between horses age < 1 year and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	22	67	14	42
10	7	21	13	39
20	0	0	4	12
40	3	9	1	3
80	0	0	1	3
160	0	0	0	0
320	1	3	0	0
640	0	0	0	0
1280	0	0	0	0
Total	33	100%	33	100%

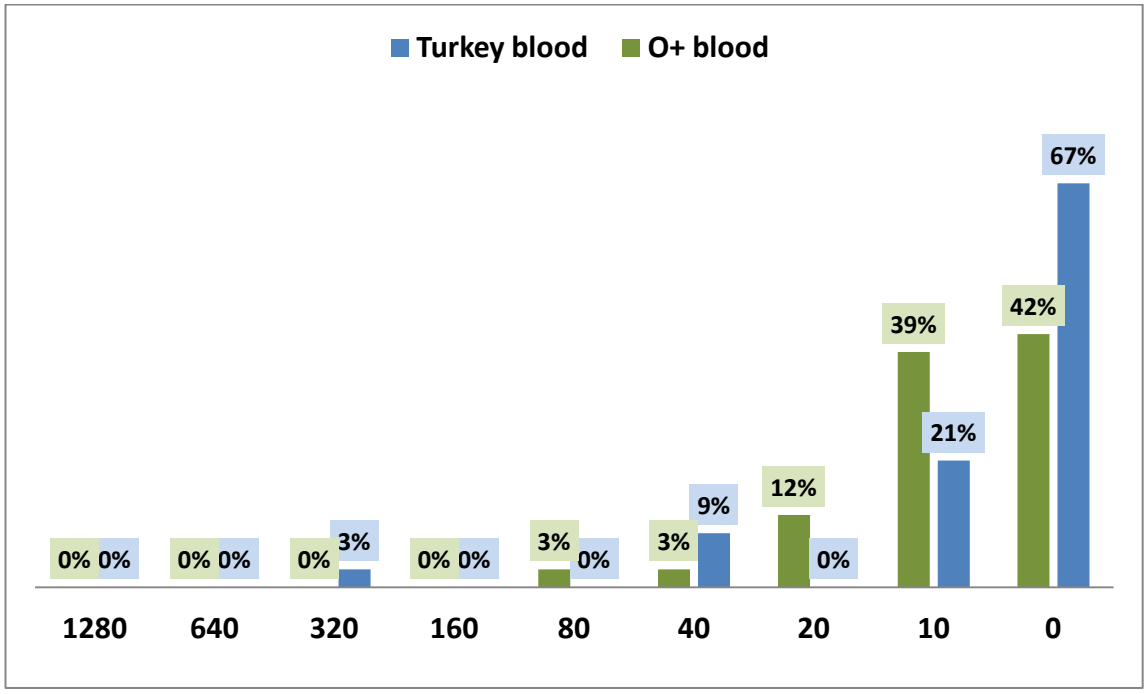

Diagram 4-4c: Relationship between horses age < 1 year and HI test

In table 4-4d, 49 samples (13%) was positive samples (titer = 10) with turkey blood and 73 samples (19%) was positive samples (titer = 10) with O+ blood, of 337 1-10 years horses.

Table 4-4d: Relationship between horses age 1-10 years and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	195	52	160	42
10	49	13	73	19
20	34	9	35	9
40	22	6	36	10
80	17	5	14	4
160	8	2	14	4
320	7	2	9	2
640	6	2	6	2
1280	39	10	30	8
Total	377	100%	377	100%

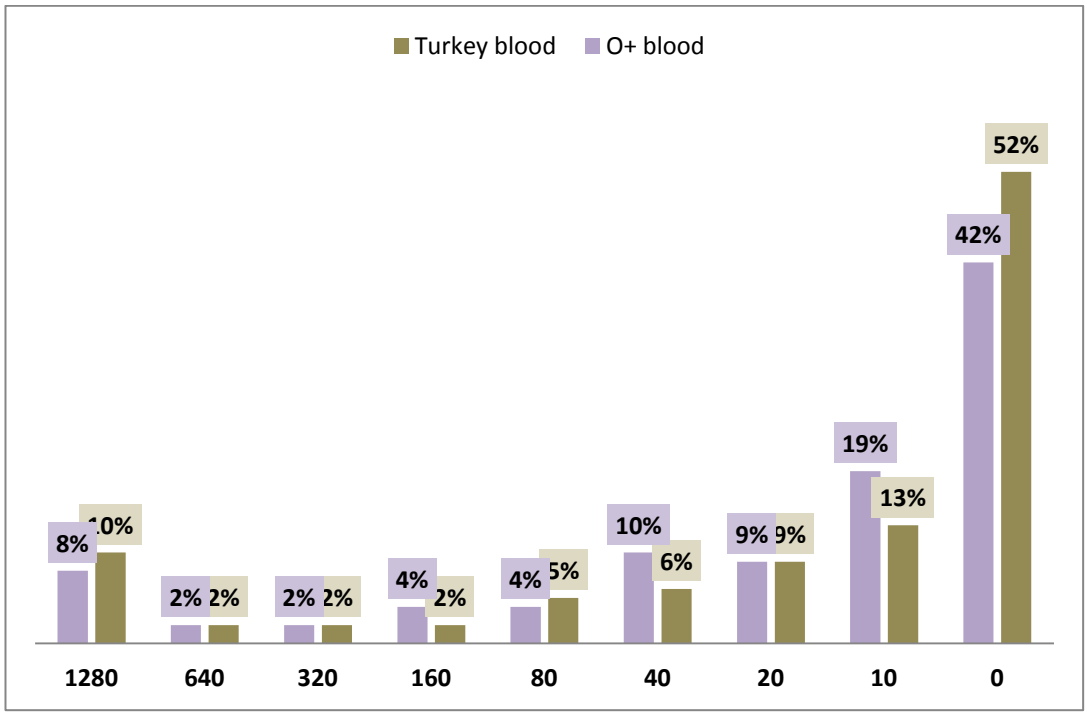

Diagram 4-4d: Relationship between horses age 1-10 years and HI test

In table 4-4e, 6 positive samples (7%) with turkey blood was (titer = 10) of 81 horse with age 11-20 years and 14 positive samples (17%) with O+ blood was (titer = 10).

Table 4-4e: Relationship between horses age 11-20 years and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	45	56	35	43
10	6	7	14	17
20	7	9	9	11
40	6	7	7	9
80	5	6	2	2
160	5	6	6	7
320	2	2	3	4
640	0	0	0	0
1280	5	6	5	6
Total	81	100%	81	100%

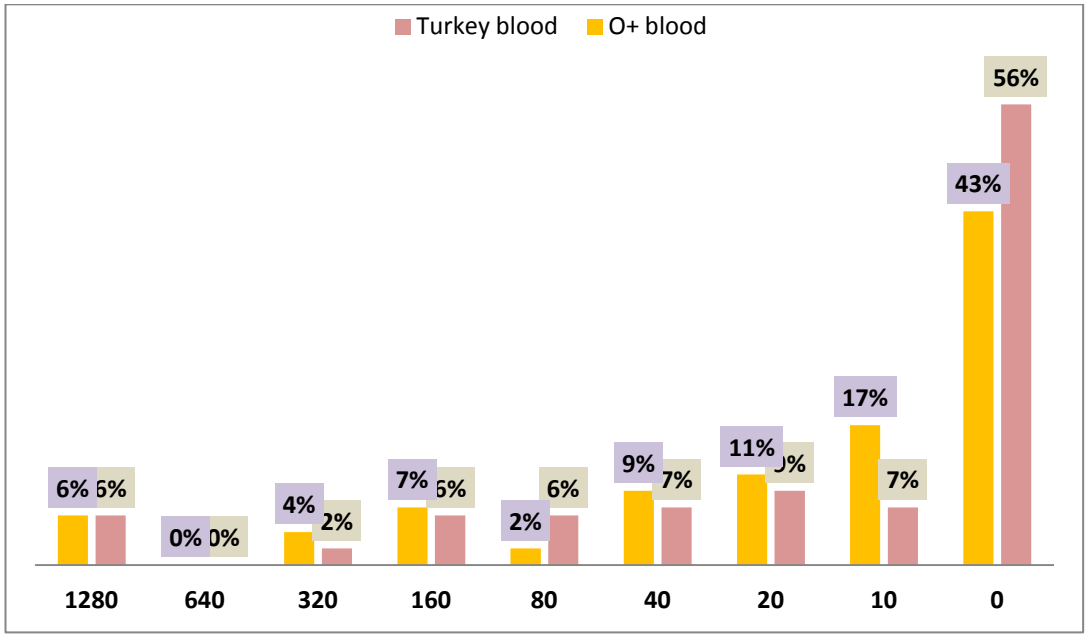

Diagram 4-4e: Relationship between horses age 11-20 years and HI test

In table 4-4f, 3 positive samples (19%) out of 16 horse >20 years (titer = 40) with turkey blood, while 4 positive samples (25%) with O+ blood for the same age.

Table 4-4f: Relationship between horses age >20 years and HI test

Hemagglutination-inhibition titer (HI)	Turkey blood		O+ blood	
	NO.	%	NO.	%
0	11	69	7	44
10	0	0	2	13
20	2	13	2	13
40	3	19	4	25
80	0	0	1	6
160	0	0	0	0
320	0	0	0	0
640	0	0	0	0
1280	0	0	0	0
Total	16	100%	16	100%

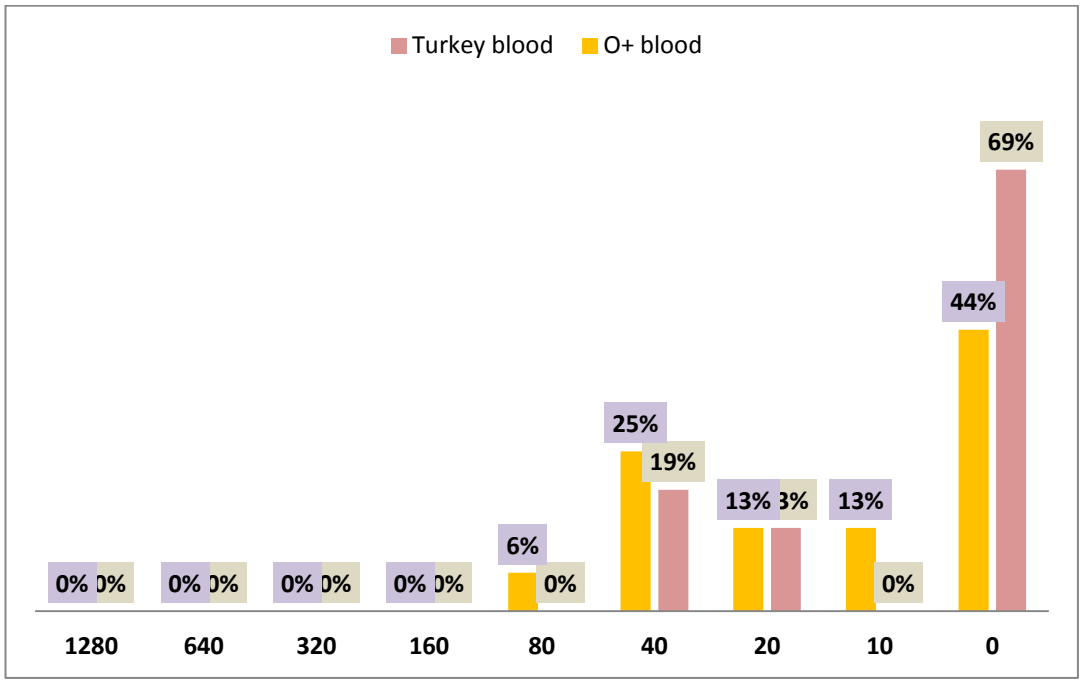

Diagram 4-4f: Relationship between horses age >20 years and HI test

The Relationship between horses age (< 1 year; 1-10 years; 11-20 years; >20 years) and HI test with turkey blood were 6.5%, 74.35%, 16% and 3.15% respectively as shown in Table 4-4g.

Table 4-4g: Relationship between horse's age and HI test with turkey blood

Age in years	Hemagglutination-inhibition titer (HI) Turkey blood									Total
	0	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	
< 1 year	22 67%	7 21%	0 0%	3 9%	0 0%	0 0%	1 3%	0 0%	0 0%	33 6.5%
1-10 years	195 52%	49 13%	34 9%	22 6%	17 5%	8 2%	7 2%	6 2%	39 10%	377 74.35%
11-20 years	45 56%	6 7%	7 9%	6 7%	5 6%	5 6%	2 2%	0 0%	5 6%	81 16%
>20 years	11 69%	0 0%	2 13%	3 19%	0 0%	0 0%	0 0%	0 0%	0 0%	16 3.15%
Total	273 100%	62 100%	43 100%	34 100%	22 100%	13 100%	10 100%	6 100%	44 100%	507 100%

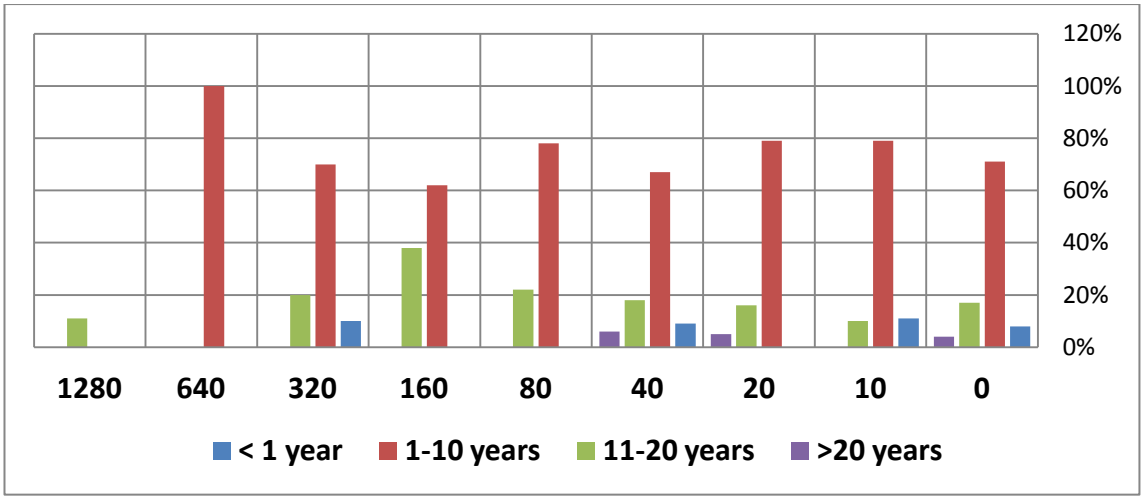

Diagram 4-4g: Relationship between horse's age and HI test with turkey blood

The Relationship between horses age (< 1 year; 1-10 years; 11-20 years; >20 years) and HI test with O+ blood were 7%, 74%, 16% and 3% respectively as shown in Table 4-4h.

Table 4-4h: Relationship between horse's age and HI test with O+ blood

Age in years	Hemagglutination-inhibition titer (HI) O+ blood									Total
	0	10	20	40	80	160	320	640	1280	
< 1 year	14	13	4	1	1	0	0	0	0	33
	42%	39%	12%	3%	3%	0%	0%	0%	0%	7%
1-10 years	160	73	35	36	14	14	9	6	30	377
	42%	19%	9%	10%	4%	4%	2%	2%	8%	74%
11-20 years	35	14	9	7	2	6	3	0	5	81
	43%	17%	11%	9%	2%	7%	4%	0%	6%	16%
>20 years	7	2	2	4	1	0	0	0	0	16
	44%	13%	13%	25%	6%	0%	0%	0%	0%	3%
Total	216	102	50	48	18	20	12	6	35	507
	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

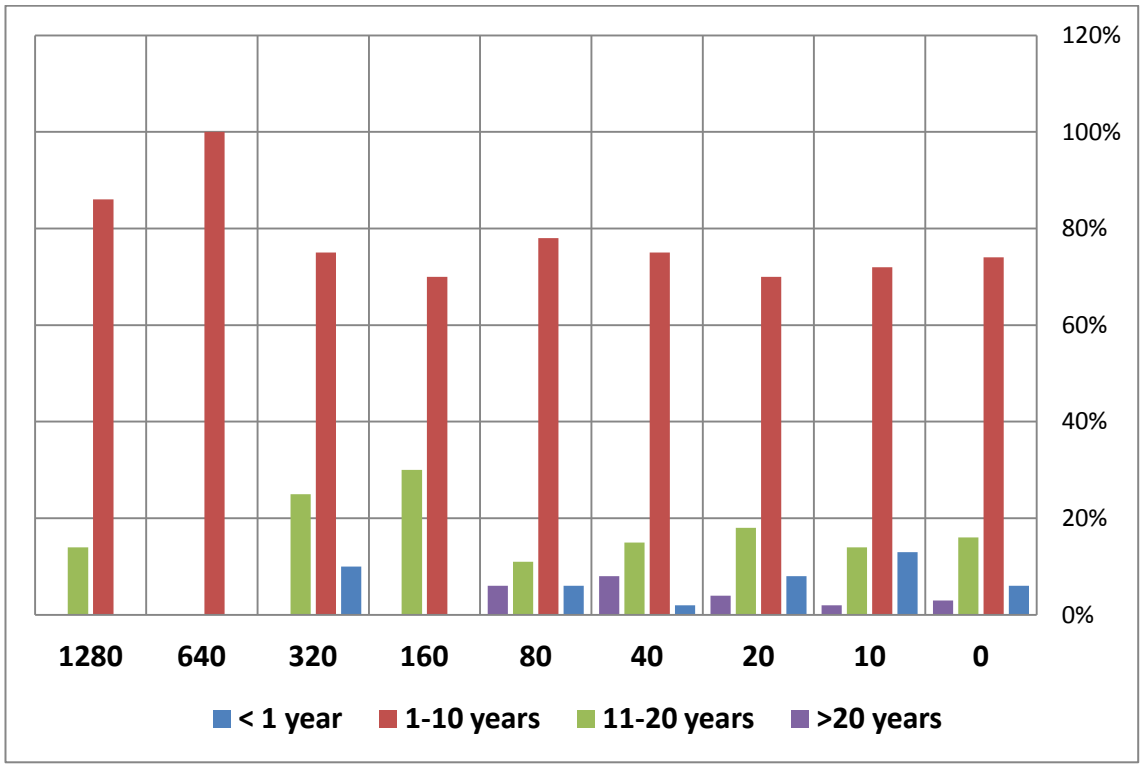

Diagram 4-4h: Relationship between horse's age and HI test with O+ blood

iii. Haemagglutination inhibition test according to Strain

The Relationship between strain (Arab, English and hybrid) and HI test with turkey blood 52%; 38% 9% respectively this was shown at Table 4-4i.

Table 4-4i: Relationship between horses strain and HI test with turkey blood

Strain	Hemagglutination-inhibition titer (HI) Turkey blood									TOTAL
	0	10	20	40	80	160	320	640	1280	
Arab strain	145 53%	20 32%	24 56%	18 55%	9 39%	5 38%	3 30%	3 50%	37 84%	264 52%
English strain	52 19%	16 26%	7 16%	4 12%	10 43%	7 54%	5 50%	2 33%	2 5%	194 38%
Hybrid strain	76 28%	26 42%	12 28%	11 33%	4 17%	1 8%	2 20%	1 17%	5 11%	46 9%
TOTAL	273 100%	62 100%	43 100%	33 100%	23 100%	13 100%	10 100%	6 100%	44 100%	507 100%

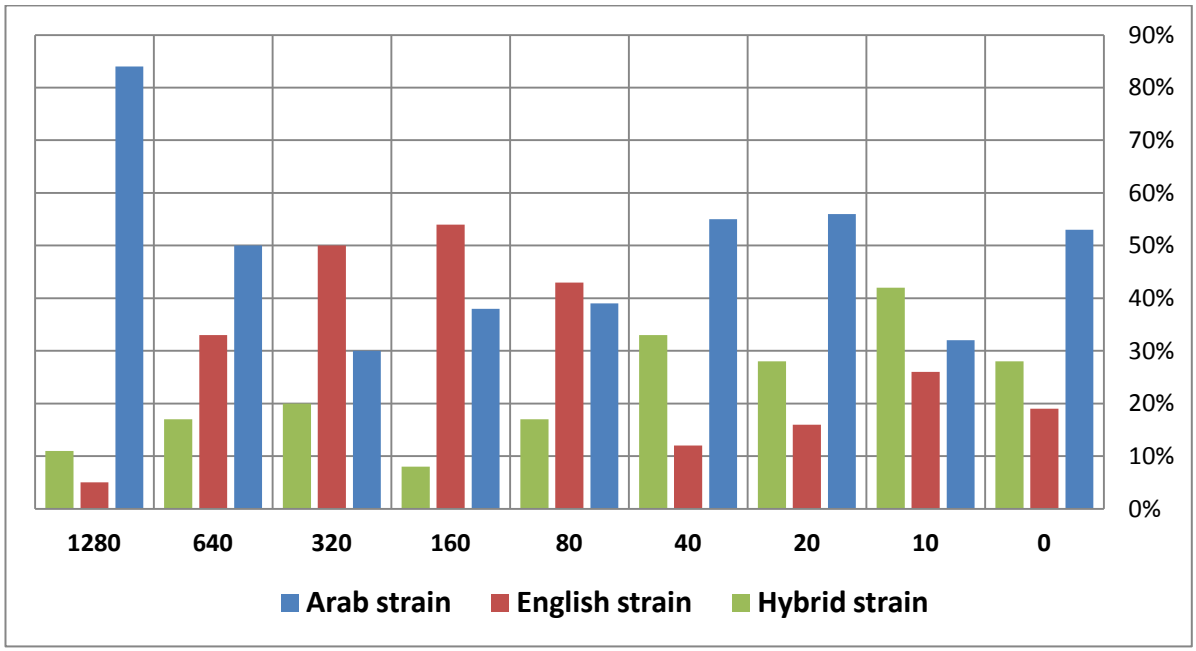

Diagram 4-4i: Relationship between horses strain and HI test with turkey blood

The Relationship between strain (Arab, English and hybrid) and HI test with O+ blood 52%, 38%, and 9% respectively this was shown at Table 4-4j.

Table 4-4j: Relationship between horses strain and HI test with O+ blood

Strain	Hemagglutination-inhibition titer (HI) O+ blood									TOTAL
	0	10	20	40	80	160	320	640	1280	
Arab strain	125 58%	31 30%	22 44%	31 66%	9 50%	11 55%	2 17%	5 83%	28 80%	264 52%
English strain	42 19%	26 25%	8 16%	5 11%	5 28%	7 35%	9 75%	1 17%	2 6%	194 38%
Hybrid strain	50 23%	45 44%	20 40%	11 23%	4 22%	2 10%	1 8%	0 0%	5 14%	46 9%
TOTAL	217 100%	102 100%	50 100%	47 100%	18 100%	20 100%	12 100%	6 100%	35 100%	507 100%

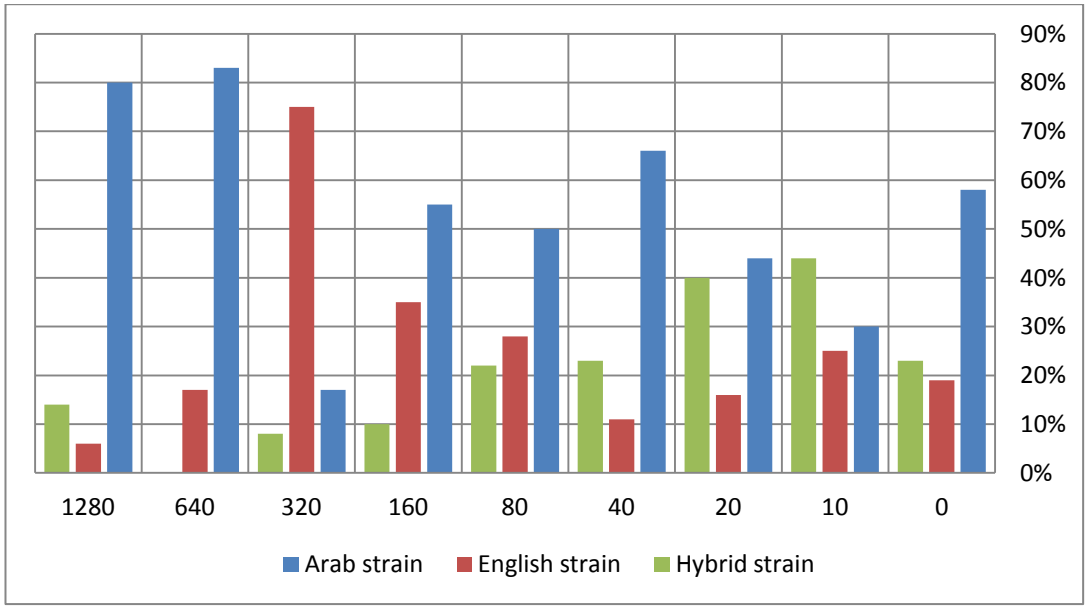

Diagram 4-4j: Relationship between horses strain and HI test with O+ blood

5. ELISA test

The ELISA test of equine influenza viruses in the all samples 507 serum studded. Founded 493 samples were positive (97%), and 14 samples were negative (3%). No significant difference was detected between the positive samples of ELISA test in Arabic strain was (98%), English strain was (98%) and in hybrid strain was (95%) as shown in Table 4-5(a, b).

Table 4-5a: Relationship between horses strain (Arabic, Hybrid and English) and ELISA test

ELISA test	Arabic strain		Hybrid strain		English strain		Total
	No.	%	No.	%	No.	%	
Positive	259	98	131	95	103	98	493
Negative	5	2	7	5	2	2	14
Total	264	100%	138	100%	105	100%	507

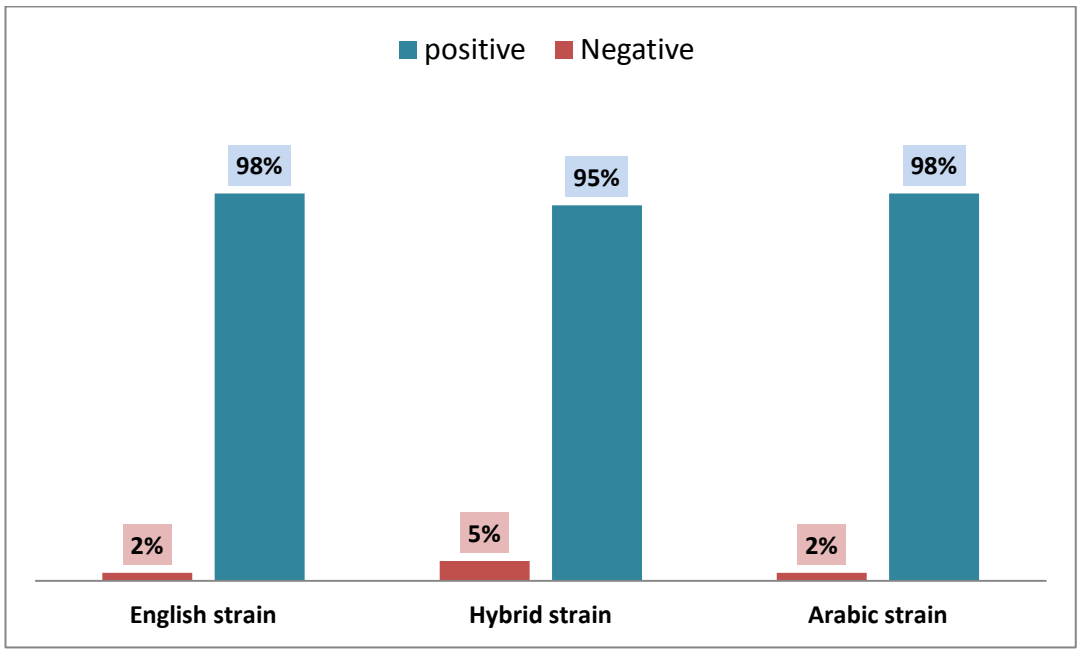

Diagram 4-5a: Relationship between horses strain (Arabic, Hybrid and English) and ELISA test

The relationship between horse gender and results of ELISA test, the positive samples of ELISA test in horse were (96%) and in mare were (98%) as shown at Table 4-5b.

Table 4-5b: Relationship between horse's gender and ELISA test

ELISA test	Horse		Mare	
	No.	%	No.	%
Positive	272	96	221	98
Negative	10	4	4	2
Total	282	100%	225	100%

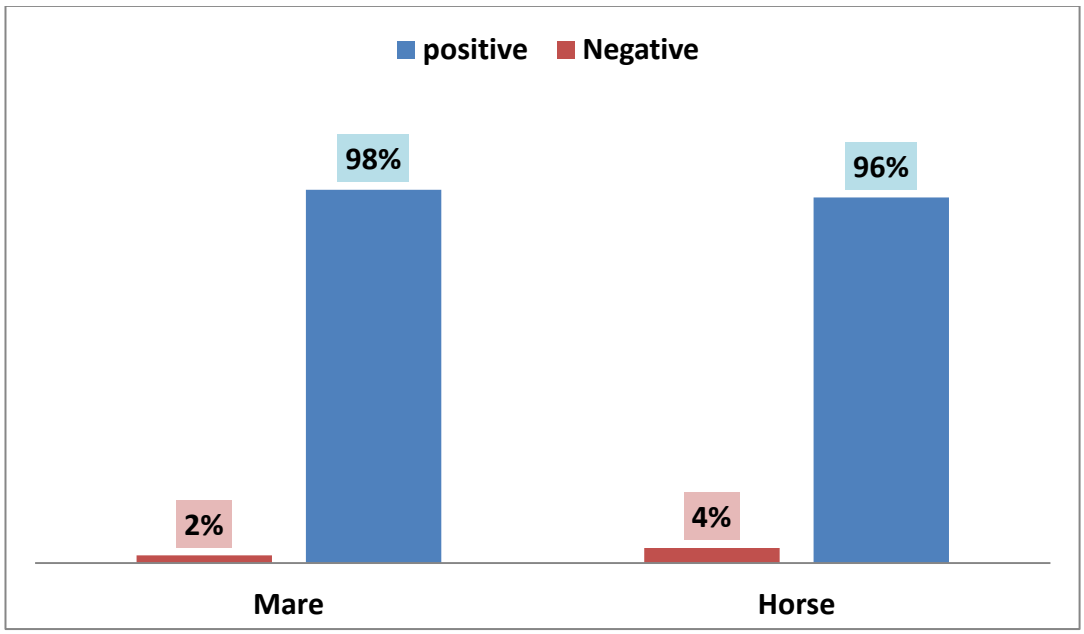

Diagram 4-5b: Relationship between horse's gander and ELISA test

6. The Relationship between HI test and ELISA test

The Relationship between HI test with turkey blood and ELISA test were **97% 3%** respectively as shown Table 4-6(a, b).

Table 4-6a: Relationship between HI test with turkey blood and ELISA test

ELISA test	Hemagglutination-inhibition titer (HI) Turkey blood									
	0	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	**total**
Positive	265 97%	61 98%	41 95%	33 100%	22 96%	13 100%	9 90%	6 100%	43 98%	493 97%
Negative	8 3%	1 2%	2 5%	0 0%	1 4%	0 0%	1 10%	0 0%	1 2%	14 3%
Total	273 100%	62 100%	43 100%	33 100%	23 100%	13 100%	10 100%	6 100%	44 100%	507 100%

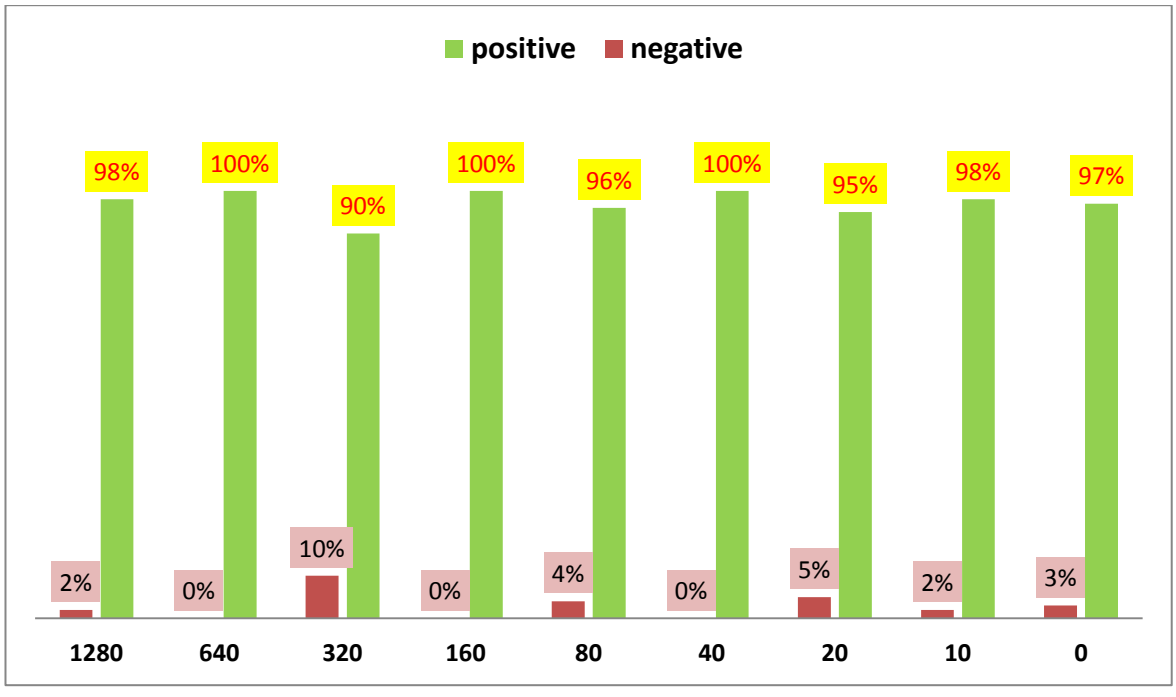

Diagram 4-6a: Relationship between HI test with turkey blood and ELISA test

The Relationship between HI test with O+ blood and ELISA test were **97%, 3%** respectively as shown Table 4-6b.

Table 4-6b: Relationship between HI test with O+ blood and ELISA test

ELISA test	Hemagglutination-inhibition titer (HI) O+ blood									Total
	0	**10**	**20**	**40**	**80**	**160**	**320**	**640**	**1280**	
Positive	264 97%	61 98%	41 95%	33 100%	23 100%	13 100%	10 100%	5 83%	43 98%	493 97%
Negative	9 3%	1 2%	2 5%	0 0%	0 0%	0 0%	0 0%	1 17%	1 2%	14 3%
Total	273 100%	62 100%	43 100%	33 100%	23 100%	13 100%	10 100%	6 100%	44 100%	507 100%

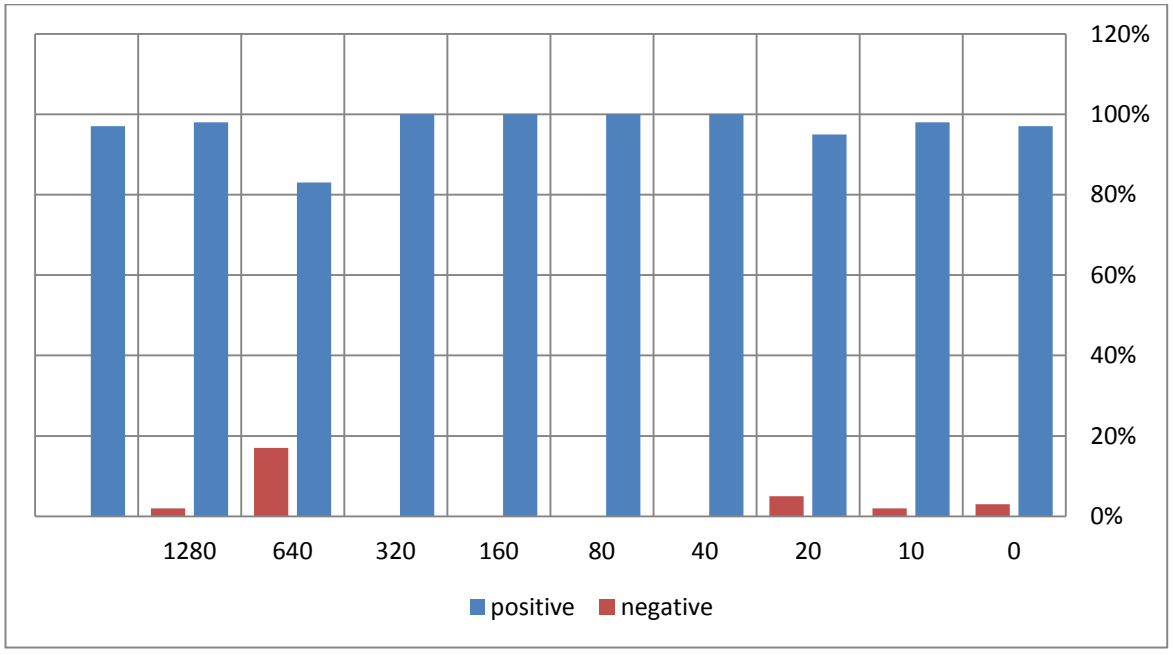

Diagram 4-6b: Relationship between HI test with O+ blood and ELISA test

7. Amplifying H7 and N7 partial sequences

Samples of equine influenza viruses available for PCR included allantoic fluids of extracts of nasal swabs from 100 horses. RNA was extracted, viral DNA was produced by reverse transcription, and PCR was performed for 35 cycles. Inactivated whole influenza vaccine was used as a positive control, and influenza B. Sichuan were used as a negative control. Amplification products were electrophoresed on 2% agarose, stained with ethidium bromide, and visualized with UV light. Results (Fig. 4-2) showed that products of about 244 bp were obtained with influenza vaccine but not with all the sample containing extracts from the horses.

8. cell culture and viral propagation

The 100 nasopharyngeal swabs isolate was propagated in MDCK cells with daily microscopic examination. But unfortunately isolates did not grow and did not appear the cytopathic effects (CPE).

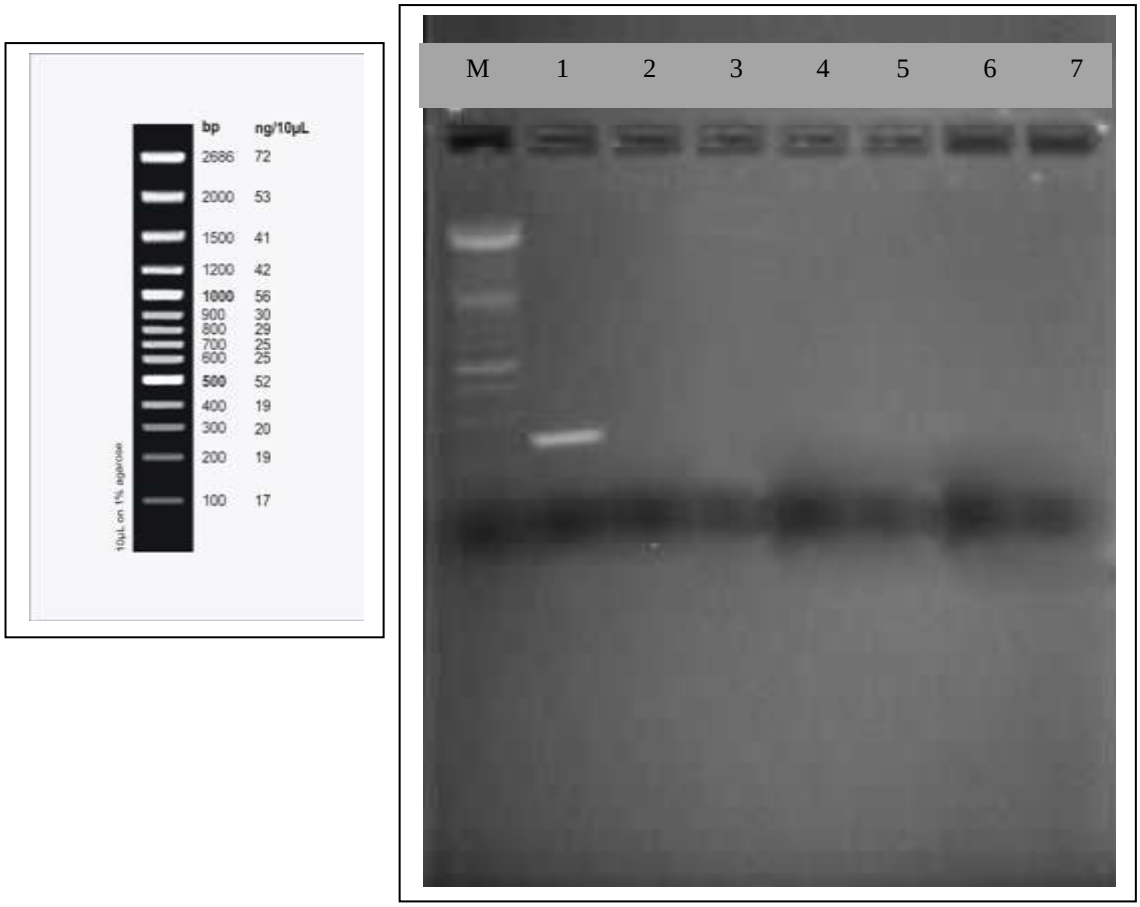

Figure 4-2: RT-PCR Amplification products of equine influenza A on an agarose gel stained with ethidium bromide, Lane M: DNA Marker ladder 100 bp. lane1: the whole virus vaccine of H7N7 (control +), lane 2 (control -), lane 3,4,5,6 and lane 7: the samples.

Chapter V

Discussion

Equine influenza virus EIV is a leading cause of respiratory disease in horses. Equine influenza infection induces a long-term immunity to re-infection. Recent strategies of vaccination aim to mimic this immunity by stimulating both antibody and cellular immune responses. Cell-mediated immunity CMI to influenza is well defined in man, but little has been done to characterize the responses in the horse. Additionally, the development of reliable assays for the measurement of equine CMI has lagged behind serological methods and vaccine development (**Paillot *et al*., 2007**).

Equine Influenza is the most economically important and most frequently diagnosed respiratory disease affecting the horse **(Landolt *et al*., 2006)**. Outbreaks of disease, considered to be influenza, have been recorded affecting horses since the 17th century. In 1872, a major epidemic of equine influenza occurred in North America. The Great Epizootic, as it was called, was first noticed near Toronto, Canada, and in 90 days had spread across the continent. It spread down the Atlantic seaboard to Havana, Cuba, whereas another branch raced west to the Pacific. The overall mortality rate in horses was probably between 1 and 2%, although in some areas up to 10% of horses were said to have died from the disease. At that time, horses were vital to the economy of North America and the outbreak forced men to pull wagons by hand, while trains and ships full of cargo sat unloaded, tram cars stood idle and deliveries of basic community essentials were no longer being made.

While few countries are dependent on the horse for transportation today, the horse is part of the social fabric of most, if not all, nations. Horses travel internationally for competition, exhibition and breeding and while such travel is ostensibly regulated by health examinations and quarantines, these are often insufficient to prevent influenza from being introduced to the resident horses. Accordingly, new variants often spread quickly to other continents, most commonly with the movement of racehorses. Despite widespread use of vaccines, most countries consider equine influenza to be endemic in its equine population although the horse populations of New Zealand and Iceland have never been affected by influenza.

As mentioned previously, the first isolation of an equine influenza virus did not occur until 1956, when a subtype H7N7 virus was isolated from the affected horses in the Czech Republic (A/Equine/1/Prague/56) **(Paul *et al.*, 2010)**. The H7N7 virus was subsequently identified as the cause of outbreaks of influenza in horses in many parts of the world. A second subtype of virus, A/Equine/2/Miami/63 H3N8 was isolated in 1963 from horses that had traveled from South America to Florida. For several years, both viruses caused equine influenza outbreaks worldwide: however, H7N7 virus has not been identified as a cause of clinical disease since the end of the 1970s. While there were some serological data in the 1990s indicating that it was still circulating in horses in Central Asia and Eastern Europe, this virus is now considered to have gone extinct in the natural environment.

While the H7N7 virus may have died out, horse populations throughout most of the world are still affected by variants of the H3N8 equine influenza virus first

isolated in 1963. This virus has evolved considerably since its first isolation and at one point two distinct lineages, American and European, existed **(Daly *et al*.,1996)**. The American lineage has since split into South American, Kentucky and Florida lineages with the Florida lineage becoming the dominant global lineage in recent years **(Lai *et al*., 2001)**. To further complicate matters, the Florida and Kentucky sublineages are reportedly evolving in parallel in the United States, such that these viruses alternately circulate in the equine population **(Lai *et al*., 2004)**.

Saudi Arabia in 2009 escalated complaints horse owners due to exposure of a number of horses for equine influenza subtype H7N7 and who moves quickly between horses, and horse owners fear exposure to significant losses. therefore it is necessary to hold immunizations for EIV prevention According to the International Organization for Animal Health OIE.

In 2002, a strain of H3N8 virus within the Florida lineage emerged in North America and quickly spread to other countries. Existing vaccines were found to be less effective against this variant **(Toulemonde *et al*., 2005)**. In 2007, this virus spread widely in Asia and was ultimately introduced into Australia later that year by infected horses from Japan through ineffective quarantine of imported horses **(Jacob, 2008)**.

Sequence comparisons indicated that six of the eight viral gene segments were closely related to avian influenza viruses **(Webster *et al*., 1992; Liu *et al*., 2009)**. Fortunately, the H3N8 Jilin virus did not appear to spread beyond China and seems to have gone extinct. It was noted at the time that the appearance of this new equine virus in China emphasized the potential for avian influenza viruses to successfully infect mammalian hosts, and that this example of species jumping

should serve as a warning for the appearance of new pandemic influenza viruses in humans **(Guo *et al*., 1992)**. This prescient warning was several years before the first case of H5N1 avian influenza in humans in 1997 **(Subbarao *et al*., 1998)**.

In the present study, from all 507 samples horses studed,493 samples were positive (97%). (**van Maanen, 2003**) reported the same finding with Elisa. 391 samples analyzed by Indirect ELISA for the rapid diagnosis of Equine Influenza (**Vila Roza *et al*., 2000**). Of these, 301 sera were positive ELISA (79%). ELISA provides a simple and rapid method of assessing specific antibodies from horse sera and offers advantages over the 'routine' HI assessments since it gives high precision, is economical of reagents and has the capacity to handle large numbers of serum samples (**Denyer *et al*., 1984**). Using hemagglutination inhibition test as standard, the ELISA showed a relative specificity and sensitivity of 83.3 and 100%, respectively (**Vila Roza *et al*., 2000**). ELISA is useful as screening test.

In this study ,titers of 1:10 or higher were recognized in HI test of the 507 samples examined (97%). **(Hiroo *et al*., 1981)** found 23 (54.8%) of the 42 horses examined by HI. They stated that HI test demonstrated good sensitivity. HI assays detect antibodies which neutralize equine influenza viruses (**Morley *et* al., 1995**). (**Jaret *et al*., 1992**) stated that these results suggest that antigenic variation in equine influenza A virus isolates and host-cell selection of antigenic variants during virus propagation may not be of sufficient magnitude to influence serological evaluation of antibody responses by hemagglutination inhibition . These findings are similar to some studies with human influenza A virus in which isolates were considered to be a mixture of virus variants. This variability has been reported to significantly alter the results of HI however other studies have shown that the

antigenic variability that occurs within and between human HINI influenza isolates is detectable with monoclonal antibodies but not with polyclonal antisera. In a previous report the tendency For equine sera to give higher HI titers when tested with cell-cultured rather than egg-cultured virus was inferred to mean that growth of influenza in eggs selected for a subpopulation of less reactive influenza variants.

In the nasal swabs from 100 horses samples with suspected viral respiratory disease were negative for equine influenza viruses by viral culture and PCR . One hundred and seventy-one nasopharyngeal swabs submitted over a two year period from cases of suspected viral respiratory disease were tested by (**Michelle *et al*., 2005**) for the presence of equine influenza virus , Virus was isolated from eight horses (4.7%). (**Mika *et* al., 2008**) Isolated equine influenza virus from 117 out of 548 nasal samples of suspected viral respiratory disease (21.3%), our negative results may be due to the small sample size.

Equine influenza virus nucleic acid was detected in 20% by PCR of nasal swabs from horses with suspected viral respiratory disease by (**Michelle *et al*., 2005**). Our negative results may be due to the small sample size and because amino acid is very sensitive.

Although cultures were more consistently positive early in infection, PCR remained positive longer (**Mika *et* al., 2008**). (**James *et al*., 1994**) have previously observed similar differences between culture and PCR or culture and ELISA in detection of influenza in humans. Apparently, culture detects smaller numbers of viable influenza viruses than does PCR, whereas PCR or ELISA can detect both nonviable and viable influenza viruses. This characteristic of PCR would obviously be valuable when prompt sampling of an outbreak is not possible. Also, PCR is

faster than culture, although more laborious. The entire process, including RNA extraction, reverse transcription, performance of PCR, and product identification can be finished within 2 days. PCR can detect influenza in frozen equine respiratory secretions (**James *et al*., 1994**). This finding has been documented previously for influenza virus in human nasal washes. (**OIE, 2008**) The present studies also showed that PCR could be carried out successfully with nasal secretions stored for several days in the refrigerator in antibiotic-containing transport medium.

PCR may be suitable with samples where influenza is no longer viable, as can be the case in epidemiologic work or with samples mailed to clinical laboratories for analysis (**James *et al*., 1994**).

(**Michelle *et al*., 2005**) study demonstrates that RT-PCR is effective in the detection of virus shedding from sera positive horses. Virus was isolated from only 15% of sera positive horses that tested positive by RT-PCR. The detection of virus shedding in subclinical infected vaccinated horses is extremely important as the introduction of such animals into an immunologically native population can lead to explosive virus spread and high morbidity.

Vaccination remains the most effective control measure for equine influenza. Vaccines need to be safe and efficacious. Vaccine efficacy is measured by the reduction in clinical signs and the decrease in virus shedding. Continuous surveillance of equine influenza virus to monitor the antigenic drift strains or novel strains is needed (**Endo *et al*., 1992**).

(**Mika *et* al., 2008**) suggested that in previous outbreak of influenza caused by the European lineage strain among vaccinated horses in Hong Kong in1992, although vaccination did not prevent 75% of the population from becoming

infected, the clinical signs of about half of the infected horses were mild (**Powell *et al.*, 1995**). In the outbreaks of influenza caused by the Florida sublineage strain among both vaccinated and unvaccinated horses in UK, the clinical signs were milder among the vaccinated horses than among immunologically naive horses (**Newton *et al.*, 2006**). In outbreak in Kanazawa Racecourse, since about half of the infected horses did not show apparent clinical signs, vaccination with the commercial inactivated vaccine was thought to contribute to reducing the morbidity rate and duration of the clinical signs. In these cases, though commendable efficiency of the vaccination was confirmed, the outbreaks of influenza caused by viruses antigenically different from vaccine strains could not prevent by the commercial vaccines. Since the antigenic disparity between the epidemic virus and vaccine strains is a significant factor for insufficient effect of inactivated vaccine, it has been suggested that equine influenza vaccines should be regularly updated (**Mumford and Wood, 1993**).

Chapter VI

Conclusions and Recommendations

6.1 Conclusions

ELISA test is suitable for the rapid diagnosis of Equine Influenza. Antibody detection remains better than viral culture or PCR for equine influenza viruses in horses with suspected viral respiratory disease.

Vaccination remains the most effective control measure for equine influenza. Vaccines need to be safe and efficacious. Vaccination with the commercial inactivated vaccine was thought to contribute to reducing the morbidity rate and duration of the clinical signs. Vaccine efficacy is measured by the reduction in clinical signs and the decrease in virus shedding. Since the antigenic disparity between the epidemic virus and vaccine strains is a significant factor for insufficient effect of inactivated vaccine, it has been suggested that equine influenza vaccines should be regularly updated. Continuous surveillance of equine influenza virus to monitor the antigenic drift strains or novel strains is needed.

6.2 Recommendations

Surveillance for influenza viruses is an urgent need for developing and developed countries. The developing countries must obtain the technology of vaccine preparation to avoid what happened in swine influenza pandemic of 2009. Researches must continue in the field of virus characterization and vaccine evaluation. This study could be considered as pilot study for the Surveillance for equine influenza viruses in KSA. Further studies for vaccine evaluation are required. Trials and investigations concern the efficacy of this vaccine against the infection with other strains and subtypes of influenza viruses should be done.

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LIST OF SYMBOL AND TERMINOLOGY

A/H1	Influenza virus type A/subtype H1
A/H3	Influenza virus type A/subtype H3
A/H5	Influenza virus type A/subtype H5
A/H7	Influenza virus A/subtype H7
A/H9	Influenza virus type/subtype H9
AI	Influenza grope A
AUSVETPLAN	Australian veterinary emergency plan
α	Alpha
bp	Base pair
C°	Conference of degree
C´	RBCs suspended in agarose containing guinea-pig complement
CCA	Chick cell agglutination
CCS	Control chicken sera
cDNA	Complementary DNA
CF	Complement fixation
CPE	Cytopathic effects
ddH_2O	Double distilled water
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's minimum essential medium
DNA	Deoxyribonucleic acid

dNTPs	Deoxy nucleotide triphosphates
EB	Ethidium bromide
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
EI	Equine influenza
EIV	Equine influenza virus
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FCS	Fetal calf serum
Flu	Influenza
g	Gravity
HA/H	Hemagglutinin
HAD	haemadsorption
H1N1	Hemagglutinin 1 and Neuraminidase 1
H3N8	Hemagglutinin 3 and Neuraminidase 8
H5N1	Hemagglutinin 5 and Neuraminidase 1
H7N7	Hemagglutinin 7 and Neuraminidase 7
HI	Hemagglutination inhibition
HK/483	Hong Kong / isolate number 483
HPAI	High pathogenic avian influenza
HP	High pathogenic
IFN	interferon
IgA	Immunoglobulin A

IgG	Immunoglobulin G
kDa	Kilo Dalton
LP	Low pathogenic
M1	Matrix protein
M2	Ion channel
MAb	Monoclonal antibody
MDCK	Madin Darby canine kidney cells
mRNA	Messenger Ribonucleic acid
NA	Neuraminidase
NA-TM	Neuraminidase – trans-membrane protein
NEP	Nuclear export protein
NP	Nucleoprotein
NS	Non Structural protein
OD	Optical density
OIE	Office International des Epizooties
OPD	Orthophenylenediamine
3P	3 plymerase protein (PB1, PB2 and PA)
PAGE	polyacrylamide gel electrophoresis
PB1, PB2, PA	Polymerases
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline-tween
PBST-FCS	Phosphate buffer saline-tween- fetal calf serum
PBS-T-BSA	Phosphate buffer saline-tween-bovine serum albumin

PCR	Polymerase chain reaction
pH	Power of hydrogen
RBCs	Red blood cells
RDE	Receptors destroying enzyme
RNA	Ribonucleic acid
RNP	Ribonucleic protein
rpm	Rotation Per Minute
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RT	real time
RT	Room temperature
S	Stock
SA	Sialic acid
SDS	Sodium dodecyl sulphate
SRH	Single radial haemolysis
ssRNA	Single strand ribonucleic acid
TAE	Tris -Acetate- EDTA buffer
TGN	Trans golgi network
TM	Trans membrane
TPCK-trypsin	Tosylamido-2-phenylethyl chloromethyl ketone
Tris-base	2-Amino-2-(hydroxymethyl)-unToi di lang thang lan trong bong toi buot gia, ve dau khi da mat em roi? Ve dau khi bao nhieu mo mong gio da vo tan... Ve dau toi biet di ve dau? http://nhatquanglan.xlphp.net/ 1,3-propanediol
Tris-HCl	2-Amino-2-(hydroxymethyl)-1,3-propanediol,

	hydrochloride
VGM	Virus growth media
VCS	Vaccinated chicken sera
VRNA	Virus ribonucleic acid
VRNP	Virus ribonucleoprotein
WHO	World Health Organization

Appendix

1- Phosphate Buffer Saline (PBS; 0.15 M; pH 7.5):

Sodium chloride (NaCl; 8.9 gm), potassium chloride (KCl; 0.2 gm), potassium dihydrogen phosphate (KH_2PO_4; 0.12 gm), disodium hydrogen phosphate (Na_2HPO_4; 0.91 gm) were dissolved in 500 ml ddH_2O and pH value was adjusted to 7.5 then the solution was brought to a final volume of 1L. The buffer was sterilized by filtration through 0.22 µm nitrocellulose membrane.

2- Diethylpyrocarbonate (DEPC ,Sigma; Deisenhofen, Germany):

DEP was diluted to a final concentration of 0.1% in water followed by overnight vigorous shaking at room temperature to allow proper mixing. To avoid inhibition of the polymerase or reverse transcriptase enzymes, excess DEPC was inactivated by autoclaving.

3- Electrophoresis buffer [Tris -Acetate- EDTA (TAE)]:

Stock solution (50X) of electrophoresis buffer was prepared containing the following ingredients:

-2M Tris base (2-amino-2-hydroxymethyl-propane-1,3-diol) 242.00 g

-1M glacial acetic acid 57.10 ml

- 0.5 M Na2 EDTA (pH 8.0) 100.0 ml

-H_2O up to one litter.

4- Loading dye (6X):

- Bromophenol blue (W/V) 0.25%

- Xylene cyanol FF (W/V) 0.25%

- Sucrose in water (W/V) 40%

5- Ethidium bromide (EB)

Ethidium bromide (EB) was prepared as 10 mg/ml stock solution by dissolving 100 mg EB in 10 ml distilled H2O, then filtered through Whatman filter paper No.1 and stored in dark bottles at room temperature.

6- DNA Marker

A 100 bp DNA step ladder (SibEnzyme Ltd, Russia) was diluted 5 times in 1X TAE before being loaded on the gel.

7- 2% agarose (GIBCO-BRL)

It was prepared in a final concentration of 2% by dissolving 2 gm agarose powder (electrophoresis grade) in 100 ml (1X) TAE buffer while heating in a microwave oven. To allow staining of the resolved nucleic acids on the gel 3µl EB were added after cooling the agarose to 56 °C at room temperature.

الملخص

في الوقت الذي مازلنا نعيش فيه توابع أزمة أنفلونزا الطيور .. وأنفلونزا الخنازير .. نقف مرة أخرى على أعتاب أزمة جديدة اسمها " أنفلونزا الخيول " التي فرضت نفسها بقوة وأصابت عدد من الخيول في المملكة العربية السعودية في سنة 2009. من المعروف أن الخيول من الحيوانات الحساسة التي تضعف مناعتها بسهولة حيث أنها تصاب بالفيروس عن طريق الاستنشاق.

وهناك العديد من الإجراءات والاحتياطات تجرى لمواجهة المرض قبل أن يتحول إلى وباء (epidemic) بين الخيول قد تمتد مخاطره للمواطنين في ظل سهولة انتقاله إلى الإنسان خاصة أن الفيروس المتسبب فيه من الفصيلة التي تصيب البشر والخنازير وهنا مكمن الخطورة.

عرفت أنفلونزا الخيول أول مرة عام 1956م، عندما تم العثور على الأنفلونزا كوباء (epidemic) واسع من الأمراض التنفسية بين الخيول في شرق أوروبا. الفيروس (A/eq/Prague/56) والذي كان H7N7 تم تعيينه كنموذج أولي (prototype) لفيروس أنفلونزا الخيول، تاريخيا يشار إليه الخيول النوع 1 Equine subtype (1) .

وآخر تفشي مؤكد حدث بسبب النوع الفيروسي H7N7 كان عام 1979 م، وفي العام 1963م قد ظهر فيروس أنفلونزا الخيول ذو نوع جيني مختلف H3N8 وصف بالخيول النوع 2 (subtype 2 equine) سبب وباء رئيسي في الولايات المتحدة الأميركية وهذا النموذج الفيروسي

(A/eq/Miami/63) قدم إلى خيول سكان فلوريدا عن طريق استيراد خيول من الأرجنتين.

أما بخصوص اللقاح (vaccination) تشير الأدلة الميدانية أن اللقاح العادي يوفر الحماية ضد العدوى H7N7، لكن اللقاح المكون من H3N8 فهو أقل فعالية. على سبيل المثال، في يناير 1976م حدث تفشي محلي من H3N8 في خيول أصيلة في نيوماركت (المملكة المتحدة) في وقت تم فيه تلقيح العديد من الخيول. فالأنفلونزا أصابت الخيول غير المحصنة وبعض من المحصنة، وتزامنت شدة المرض مع الفترة التي تلت التطعيم. فهناك إسطبلات فيها أكثر من 75% من الخيول المحصنة لم تتأثر بخطورة.

بين العام 1978م والعام 1981م، تم تسجيل تفشي واسع الانتشار من فيروسات H3N8 في أوربا وشمال أمريكا مع عدوى حدثت في الخيول المحصنة وغير المحصنة. الأنفلونزا اقتصرت على الخيول غير المحصنة خلال الستة أشهر الأولى من السنة، ولكنها امتدت إلى الخيول الأصيلة المحصنة في يونيو 1979م، مما يوفر دليل واضح أن اللقاحات لا توفر مناعة (immunity) ضد العدوى لسنة كاملة، حتي مع " الجرعات التنشيطية booster doses – " تأثرت السباقات فحدثت خسائر هائلة، مما أدى إلى جعل اللقاح المحتوي على السلالتين H7N7 وH3N8 إلزامي في المملكة المتحدة وايرلندا في عام 1981م.

ثم في العام 1989م، ظهر وباء رئيسي لأنفلونزا H3N8 في أوربا أصاب ليس فقط الخيول غير المحصنة ولكن عدد كبير من الخيول المحصنة. وانتشرت في

أسيا وتسببت في إصابة نحو 80% ونفوق نحو 20% من الخيول في منطقة شمال الصين ووجد أن أنتجينات هذه العثرة (A/eq/jilin/89) أقرب للعثرة المنتشرة في البط عن العثرة المنتشرة في الخيول.

ظهور المرض بين الحالات المحصنة يدعو إلى دراسة جادة حول اللقاح ومدى فعاليته وتأثيره (vaccine potency) على السلالات المسببة للمرض عن طريق التجارب المثبتة والمستخدمة في هذه الحالات.

فخلال الخمسة عشر سنة الأخيرة، تم تسجيل عدد من حالات التفشي الخطيرة لأنفلونزا H3N8 في الخيول المحلية بدون تاريخ سابق للمرض، وثبت انتشار المرض حول العالم في عدد كبير من الدول.

وأخيرا وليس أخرا ظهرت العدوى في عدد من خيول المملكة العربية السعودية في المنطقة الشرقية في نهاية 2008 وبداية 2009 وخصوصا في إسطبلات محافظة القطيف بعد أن أصابت إسطبلات في الجبيل. منبع هذا التفشي يمكن إرجاعه إلى نقل الخيول المصابة من مناطق كانت فيها الأنفلونزا كوباء، ونقص الحجر الصحي في ميناء الدخول يسمح بقدوم الخيول المصابة إلى الخيول المحلية مما يؤدي إلى انتشار الأمراض والوفيات.

في هذه الدراسة، أخذت 507 عينة من الخيل تحت الدراسة منها 493 عينة اعطت نتيجة ايجابية في اختبار الانزيم المناعي (Elisa test) حيث اظهر هذا الاختبار مدى دقته وحساسيته في الفحص.

وعند فحص 507 عينة بواسطة اختبار تثبيط التلازن (HI) اظهر مانسبته (97%) منها نتيجة ايجابية.

وقد تم فحص 100 عينة من المسحات الانفية بواسطة اختبار سلسلة تفاعل بلمرة النسخ العاكس وتنميتها علي مزارع الانسجة لخلايا MDCK (madin-darby canine kidney) والتي اعطت نتيجة سلبية بالنسبة للانتجين.