Equine Influenza Virus: Characterisation, Epidemiology and Vaccination

Thesis presented by
Sarah Gildea

University of Limerick
Ollscoil Lumnigh

Student: Sarah Gildea
Student ID: 9851976
Date: October 2011
Supervisors: Professor Sean Arkins, Professor Ann Cullinane

Submitted for the award of Doctor of Philosophy at the University of Limerick
Abstract

The epidemiology, characterisation and vaccination responses to equine influenza (EI) in Ireland were studied between 2007 and 2010. Epidemiological evidence indicated a seasonal pattern to outbreaks with 50% and 39% occurring over the summer and winter months, respectively. Outbreaks were recorded in 13 of the 32 counties and the majority of cases occurred following animal movement on or off premises. Other key influences included housing type and fomites/personnel. Epidemiological evidence revealed poor vaccine compliance on premises where horses were infected with EI, while on premises where vaccination was widely practiced, the duration of vaccination history and time since last vaccination influenced disease expression. In a population of 1149 horses/ponies with mixed levels of antibody, 34%, 11%, 32% and 23% were considered to have index case potential, partial protection, clinical protection and virological protection respectively. Younger animals were identified as most susceptible to EI and the majority of teaser stallions had little serological evidence of vaccination. Phylogenetic analysis of the HA gene of 18 EI viruses identified suggested that predominant strains circulating among Irish horses were from clade 1 and clade 2 of the Florida sublineage. Equine influenza vaccine efficacy was examined in two different equine populations, mature National Hunt (NH) horses and immunologically naïve Thoroughbred (TB) weanlings. There was no significant difference between the levels of antibody response induced following booster vaccination with any of the six vaccines in the NH population. While the mean SRH levels post booster vaccination suggested that this population was clinically protected, analysis of the individual responses suggested that there was potential for vaccine breakdown among some horses. Antibody responses of weanlings vaccinated with the whole virus vaccine Duvaxyn IET Plus were significantly higher than those of the horses vaccinated with any of the other products. A high incidence of poor responders was observed post first vaccination (V1). Vaccination of weanlings which had maternally-derived antibodies effectively primed the animals but in some cases their response to the primary course was lower than that of seronegative weanlings. Overall, the results indicated that EI is endemic in Ireland. Vaccine compliance is good within certain sectors of the industry however, there are vulnerable subgroups where vaccination is poorly executed thus creating potential pockets of disease and facilitating virus spread.
Author’s Declaration

I hereby declare that this thesis has not been previously submitted to this or any other institution. It is entirely my own original work and where the use or reference has been made to the work of any other persons, it has been fully acknowledged and properly referenced.


Technical staff in the Virology Unit at the Irish Equine Centre assisted with diagnostic testing.


The molecular epidemiology of equine influenza in Ireland from 2007-2010 and its international significance. Accepted for publication by: Equine Veterinary Journal.

The sequencing of the HA gene for EI viruses detected in Ireland in 2007 was carried out by Dr. Michelle Quinlivan at the Irish Equine Centre.


A comparative antibody study of the potential susceptibility of Thoroughbred and non-Thoroughbred horse populations in Ireland to equine influenza virus. Influenza and Other Respiratory Viruses, 4(6), 363-72.

A comparison of antibody responses to commercial equine influenza vaccines following annual booster vaccination of National Hunt Horses - a randomised blind study. Vaccine, 29(22), 3917-22.

Statistical analysis was carried out by Dr. Cathal Walsh (Trinity College, Dublin)


A comparison of antibody responses to commercial equine influenza vaccines following primary vaccination of Thoroughbred weanlings – a randomised blind study. Accepted for publication by: Vaccine

Statistical analysis was carried out by Dr. Cathal Walsh (Trinity College, Dublin)

Sarah Gildea

Dated: November 2011
Acknowledgements

First and foremost, I would like to thank Professor Ann Cullinane for the opportunity to carry out this work and for all her time and patience. The joy and enthusiasm she has for research was contagious and motivational for me even during tough times in my PhD pursuit.

I would also like to thank Professor Sean Arkins for his guidance, constructive criticism and sound advice. His corrections and comments on my manuscripts have been a continuous source of humour to me even in times of despair.

I am deeply grateful to the Department of Agriculture without whose support and funding this work would not have been possible. I would also like to express special thanks to all the staff in the Virology Unit at the Irish Equine Centre especially Dr. Michelle Quinlivan for her assistance.

I would like to thank my parents for the tremendous sacrifices they have made for me down through the years. They have been a constant source of encouragement, support and wisdom. Most importantly, they have consistently helped me keep in perspective what is important in life.

To Paul, thank you for your kindness, patience and emotional support, but most importantly, thank you for putting up with me.

Finally, this thesis would not have been possible without the assistance of many different veterinary clinicians, trainers and owners throughout the country. These people are too numerous to mention, however I am nonetheless, very grateful.
# Table of Contents

Abstract.................................................................................................................................i

Author’s Declaration ................................................................................................................ii

Acknowledgements..................................................................................................................iv

Table of Contents ....................................................................................................................v

Table of Figures ........................................................................................................................x

List of Tables ..........................................................................................................................xii

Chapter 1 ..................................................................................................................................1

Literature Review .....................................................................................................................1

1.1 Introduction .......................................................................................................................2

1.2 Objectives ..........................................................................................................................2

1.3 Aetiology ............................................................................................................................3

1.4 Epidemiology .....................................................................................................................8

1.5 Clinical signs and transmission .........................................................................................16

1.6 Pathogenesis .....................................................................................................................17

1.7 Diagnosis ..........................................................................................................................19

1.8 Prevention, management and control ...............................................................................22

1.9 Surveillance and phylogenetic analysis .............................................................................27

1.10 Interspecies transmission ...............................................................................................30

1.11 Zoonotic potential of EIV .............................................................................................33

1.12 Summary ..........................................................................................................................34

1.13 References .......................................................................................................................35

Chapter 2 ..................................................................................................................................52

Management and environmental factors involved in Equine Influenza outbreaks in Ireland from 2007 to 2010 .........................................................................................52

2.1 Abstract .............................................................................................................................53
3.4 Results .......................................................................................................................... 89
  3.4.1 Genetic characterisation and phylogeny ................................................................. 89
  3.4.2 Amino acid alignment ............................................................................................ 93
3.5 Discussion .................................................................................................................. 96
3.6 References ................................................................................................................ 99

Chapter 4 ......................................................................................................................... 104
A Comparative Antibody Study of the Potential Susceptibility of Thoroughbred and Non-Thoroughbred Horse Populations in Ireland to Equine Influenza Virus ................................................................. 104
  4.1 Abstract .................................................................................................................... 105
  4.2 Introduction ............................................................................................................. 106
  4.3 Materials and Methods ............................................................................................ 108
     4.3.1 Samples collection and horse populations ......................................................... 108
     4.3.2 Serology ........................................................................................................... 109
     4.3.3 Statistical analysis ............................................................................................ 109
  4.4 Results ..................................................................................................................... 110
     4.4.1 TB weanlings: ................................................................................................. 115
     4.4.2 TB yearlings ..................................................................................................... 116
     4.4.3 TB broodmares ................................................................................................. 117
     4.4.4 Teaser Stallion population .............................................................................. 118
     4.4.5 Thoroughbred horses in training ...................................................................... 119
     4.4.6 Non-Thoroughbred population ...................................................................... 120
  4.5 Discussion ................................................................................................................ 121
  4.6 References ................................................................................................................ 127

Chapter 5 ........................................................................................................................................... 132
A comparison of antibody responses to commercial equine influenza vaccines following annual booster vaccination of National Hunt Horses – a randomised blind study ................................................................................................................................. 132
Chapter 5 .......................................................................................................................... 133

5.1 Abstract .......................................................................................................................... 133

5.2 Introduction ..................................................................................................................... 134

5.3 Materials and Methods .................................................................................................. 135

5.3.1 Horses ......................................................................................................................... 135

5.3.2 Vaccines ...................................................................................................................... 135

5.3.3 Vaccinations ............................................................................................................... 137

5.3.4 Collection of samples ................................................................................................. 137

5.3.5 Serology ...................................................................................................................... 137

5.3.6 Statistical analysis ...................................................................................................... 138

5.4 Results ............................................................................................................................ 139

5.4.1 Course of antibody response ....................................................................................... 139

5.4.2 Comparison of antibody response ............................................................................. 139

5.4.3 Failure or delay in H3N8 response to vaccination ....................................................... 144

5.4.4 Age of horse ................................................................................................................. 145

5.5 Discussion ........................................................................................................................ 146

5.6 References ..................................................................................................................... 150

Chapter 6 .......................................................................................................................... 154

A comparison of antibody responses to commercial equine influenza vaccines following primary vaccination of Thoroughbred weanlings– a randomised blind study .......................................................................................................................... 154

6.1 Abstract .......................................................................................................................... 155

6.2 Introduction ..................................................................................................................... 156

6.3 Materials and Methods .................................................................................................. 159

6.3.1 Horses ......................................................................................................................... 159

6.3.2 Vaccines ...................................................................................................................... 159

6.3.3 Vaccination .................................................................................................................. 161

6.3.4 Collection of samples ................................................................................................. 161

6.3.5 Serology ...................................................................................................................... 161
6.3.6 Statistical analysis ................................................................. 162
6.4 Results .................................................................................. 163
  6.4.1 Course of antibody response .............................................. 163
  6.4.2 Comparison of antibody response ..................................... 167
  6.4.3 Area under curve (AUC) .................................................... 169
  6.4.4 Maternally derived antibodies .......................................... 171
  6.4.5 Failure or delay in response to vaccination ...................... 172
  6.4.6 Effect of premises, gender, age of foal at the time of V1 and age of mare on antibody responses ........................................ 175
6.5 Discussion ............................................................................. 175
6.6 References ............................................................................ 182

Chapter 7 ..................................................................................... 189
Discussion and Conclusion ............................................................ 189
  7.1 Discussion ........................................................................... 190
  7.2 Conclusions .......................................................................... 193
  7.3 Future Work .......................................................................... 196
  7.4 References ............................................................................ 199
# Table of Figures

**Figure 1.1**: Structure of influenza A virus ................................................................. 5  
**Figure 1.2**: Three dimensional structure of the H3 subtype HA protein ......................... 7  
**Figure 1.3**: Equine Influenza Virus H3N8 Phyloigentic Tree constructed using HA1 nucleotide sequence data .................................................................................. 11  
**Figure 1.4**: Equine Influenza Activity (July – December 2007) ........................................... 12  
**Figure 1.5**: Equine Influenza Activity (January - June 2008) .............................................. 13  
**Figure 1.6**: Equine Influenza Activity (July - December 2008) ............................................. 13  
**Figure 1.7**: Equine Influenza Activity (January - June 2009) ............................................. 14  
**Figure 1.8**: Equine Influenza Activity (July -December 2009) ........................................... 14  
**Figure 1.9**: Equine Influenza Activity (January - June 2010) ............................................ 15  
**Figure 1.10**: Equine Influenza Activity (July -December 2010) ........................................... 15  
**Figure 1.11**: Illustration of Influenza A virus infecting host cell ......................................... 18  
**Figure 1.12**: Steps in isolation of EIV in embryonated hens eggs ........................................ 20  
**Figure 1.13**: Zones of Haemolysis as measured by the SRH test ....................................... 22  
**Figure 1.14**: The reservoir of influenza A viruses ............................................................. 30  
**Figure 1.15**: Phylogenic tree of CIV and EIV ..................................................................... 33  
**Figure 2.1**: Geographical Distribution (by county) of premises affected by EI from June 2007 to January 2010 .................................................................................. 60  
**Figure 2.2**: Pattern of EIV clinical signs on Premises 2 .................................................... 64  
**Figure 2.3**: Pattern of EIV clinical signs on Premises 9 ..................................................... 66  
**Figure 3.1**: Location of the primers listed in Table 3.1 with reference to a schematic diagram of the HA gene ............................................................................................ 87  
**Figure 3.2**: Phylogenetic tree of HA1 nucleotide sequences (n=18) ................................... 91  
**Figure 3.3**: HA1 amino acid sequence alignment ............................................................ 95  
**Figure 4.1**: Mean H3N8 antibody level of weanlings, yearlings, broodmares, teasers, horses in training and non-Thoroughbreds ................................................................. 112  
**Figure 4.2**: Mean H7N7 antibody level of weanlings, yearlings, broodmares, teasers, horses in training and non-Thoroughbreds ................................................................. 113  
**Figure 4.3**: Level of H3N8 Antibody Protection in Weanlings ............................................. 116  
**Figure 4.4**: Level of H3N8 Antibody Protection in Yearlings ............................................. 117  
**Figure 4.5**: Level of H3N8 Antibody Protection in Broodmares ......................................... 118
Figure 4.6: Level of H3N8 Antibody Protection in Teasers ........................................119
Figure 4.7: Level of H3N8 Antibody Protection in Horses in Training ......................120
Figure 4.8: Level of H3N8 Antibody Protection in non-Thoroughbreds ......................121
Figure 5.1: SRH antibody response (mm²) to A/eq/Prague/56 in the weeks following booster vaccination (Data are means +/- SEM) .................................................................140
Figure 5.2: SRH antibody response (mm²) to A/eq/Kildare/92 in the weeks following booster vaccination (Data are means +/- SEM) .................................................................141
Figure 5.3: SRH antibody response (mm²) to A/eq/Newmarket/2/93 in the weeks following booster vaccination (Data are means +/- SEM) .................................................................142
Figure 5.4: SRH antibody response (mm²) to A/eq/South Africa/4/03 in the weeks following booster vaccination (Data are means +/- SEM) .................................................................143
(Virological protection) .........................................................................................................144
Figure 5.5: The relationship between the SRH antibody levels on the day of vaccination (DV) and response to booster vaccination .................................................................145
Figure 6.1: Mean SRH antibody in vaccinated weanlings measured against A/eq/Kildare/92 .................................................................................................................................164
Figure 6.2: Mean SRH antibody in vaccinated weanlings measured against A/eq/Newmarket/2/93 .................................................................................................................................165
Figure 6.3: Mean SRH antibody in vaccinated weanlings measured against A/eq/South Africa/4/03 .................................................................................................................................166
Figure 6.4: Mean H3N8 SRH antibody levels in vaccinated weanlings with MDA at the time of V1 .................................................................................................................................172
List of Tables

Table 1.1: Segments of Equine Influenza Virus Genome ......................................................... 4
Table 1.2: Antigenic regions on the HA1 subunit protein............................................................. 6
Table 1.3: Amino acid substitutions “diagnostic” of the division between recent American and European isolates .................................................................................................................. 9
Table 2.1: EI in Ireland from 2007 to 2010; Premises number, date, location, method of detection, type of premises and reported vaccination status of animals ................................. 58
Table 2.2: Premises number, vaccination status, housing type, morbidity and clinical signs on premises where epidemiological investigations took place ........................................ 61
Table 2.3: Premises number, vaccination status and range of ages, average age of clinically affected and average age of healthy cohorts on 11 premises ............................................. 67
Table 2.4: SRH data for confirmed cases of EI on initial sampling............................................. 68
Table 3.1: Primer sequences ............................................................................................................... 86
Table 3.2: EI viruses included in phylogenetic analysis ................................................................. 88
Table 3.3: EI viruses detected in Ireland 2007-2010: Type of premises and reported vaccination status of horses .................................................................................................................. 92
Table 4.1: Descriptive Statistics of H3N8 and H7N7 antibody results ........................................ 111
Table 4.2: Distribution of EI H3N8 SRH levels in selected populations ........................................ 114
Table 4.3: Distribution of EI H7N7 SRH levels in selected populations ........................................ 114
Table 4.4a: Distribution of EI H3N8 and H7N7 SRH levels on weanling, yearling and broodmare premises (≥1 of 5 horses tested on each premises) .................................................. 115
Table 4.4b: Distribution of EI H3N8 and H7N7 SRH levels on weanling, yearling and broodmare premises (all horses (5) tested on each premises) ......................................................... 115
Table 5.1: Vaccine product details .................................................................................................. 136
Table 5.2 ......................................................................................................................................... 137
Timetable for vaccination and collection of samples from horses .................................................. 137
Table 5.3: Influence of pre-existing H3N8 antibody levels on booster vaccination response ........................................... 144
Table 6.1: Vaccine product details .................................................................................................. 160
Table 6.2 ......................................................................................................................................... 161
Timetable for vaccination and collection of samples from horses .................................................. 161
Table 6.3: Post hoc Tukey’s HSD comparisons .............................................................................. 167
Table 6.4: Tukey’s HSD of antibody response between vaccines at different sampling time points........................................................................................................................................... 169

Table 6.5: Tukey’s HSD multiple comparisons of mean AUC....................................................... 169

Table 6.6: Mean H3N8 SRH antibody levels for weanlings with MDA, poor responders and normal weanlings ........................................................................................................................................... 174

Table 6.7: Number of poor responders to each vaccine ................................................................. 175
Chapter 1

Literature Review
1.1 Introduction
Despite being first isolated from horses over 50 years ago, equine influenza virus (EIV) remains a major cause of respiratory disease in horses worldwide (Timoney, 1996). The continued evolution of the virus through antigenic drift together with an increase in the international transport of horses has resulted in frequent outbreaks of the disease with significant financial impact. Equine influenza (EI) is highly contagious and spreads rapidly within susceptible populations. With the exception of New Zealand and Iceland, outbreaks of EI have occurred worldwide and in many countries the virus is endemic. In countries where EI is endemic, disease control relies heavily on mandatory vaccination of high risk populations. The success however, of such mandatory vaccination programmes is largely dictated by commercially available vaccines containing epidemiologically relevant strains and being efficacious in providing both clinical and virological protection. The study focuses on the epidemiology and characterisation of EIV in Ireland and on the antibody response to commercially available vaccines among different equine populations. The research chapters are presented in research journal format, as published.

1.2 Objectives
The overall objectives of this study were as follows:

1) To identify key management and environment factors that determine the risk of horses contracting EI in an EIV endemic country and to identify appropriate control strategies.

2) To carry out genetic characterisation of EI viruses identified in Ireland between 2007 and 2010.

3) To establish which groups within the Irish equine population are most susceptible to EIV by quantifying their antibodies against the virus.

4) To carry out an independent comparison of antibody responses to commercially available EI vaccines following annual booster vaccination in a population of National Hunt horses in training.

5) To carry out an independent comparison of antibody responses to commercially available EI vaccines following primary vaccination (V1, V2, V3) in a population of immunologically naive Thoroughbred weanlings.
1.3 Aetiology

Equine influenza virus is a member of the family *Orthomyxoviridae*, which are enveloped viruses with segmented, single stranded, negative sense ribonucleic acid genomes. Equine influenza is characterised as a genus Influenza A virus on the basis of antigenic properties of the internal nucleoprotein (NP) and matrix (M) proteins (Lamb and Krug, 2001). In contrast to Influenza A viruses, which infect a wide variety of species (humans, horses, birds, pigs), Influenza B viruses and Influenza C viruses have also been identified, however, with limited host range. Influenza B viruses have been documented as affecting only humans and seals (Osterhaus et al., 2000) while Influenza C viruses are known to infect humans and pigs (Guo et al., 1983). Such limited host range may be responsible for the lack of Influenza B and Influenza C pandemics in humans in contrast with those caused by Influenza A viruses (Matsuzaki et al., 2004). Influenza A and B viruses each contain eight distinct RNA segments (Table 1.1) whereas influenza C viruses contain only seven. The segmented nature of the viral genome is a critically important feature of the influenza A viral structure. In the event that cells are infected with two or more different influenza A viruses, the exchange of RNA segments between the viruses permits the generation of progeny viruses containing novel combinations of genes (antigenic shift). Such genetic reassortment could facilitate the transmission of influenza virus to other species whereby extend the virus host range.
Table 1.1: Segments of Equine Influenza Virus Genome

<table>
<thead>
<tr>
<th>Segment</th>
<th>Length (nt)</th>
<th>Encoded Protein</th>
<th>Name of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,341</td>
<td>PB2</td>
<td>Polymerase B2</td>
</tr>
<tr>
<td>2</td>
<td>2,341</td>
<td>PB1</td>
<td>Polymerase B1</td>
</tr>
<tr>
<td>3</td>
<td>2,233</td>
<td>PA</td>
<td>Polymerase A</td>
</tr>
<tr>
<td>4</td>
<td>1,762</td>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>5</td>
<td>1,565</td>
<td>NP</td>
<td>Nucleoprotein</td>
</tr>
<tr>
<td>6</td>
<td>1,462</td>
<td>NA</td>
<td>Neuraminidase</td>
</tr>
<tr>
<td>7</td>
<td>1,027</td>
<td>M</td>
<td>Matrix</td>
</tr>
<tr>
<td>8</td>
<td>890</td>
<td>NS</td>
<td>Non-structural</td>
</tr>
</tbody>
</table>

Adapted from Lamb and Krug, Fields Virology, 2001

The eight RNA segments of influenza A virus typically encode for 11 or 12 viral proteins (Palese and Shaw, 2007). These include the non-structural proteins PB1, PB2, PA, NS1, nuclear export protein (NEP) also known as NS2 and the newly identified N40 protein which is expressed from the PB1 segment (Wise et al., 2009). The RNA segments also encode for the structural proteins nucleoprotein (NP), matrix proteins (M1 and M2), haemagglutinin (HA) and neuraminidase (NA). In addition, some select influenza A viruses express the protein PB1-F2. This protein was first discovered by Chen et al., (2001) and has been demonstrated to impact host defence mechanisms which in turn enhances pathogenicity in vivo (Zamarin et al., 2006). An illustration of the structure of influenza A virus is shown in Figure 1.1.
The lipid envelope of virion contains approximately 400-500 HA and 100 NA protruding spikes (Ruigrok et al., 1984). The HA, a major surface glycoprotein, was originally named because of its ability to agglutinate erythrocytes (Hirst, 1941) by attachment of specific sialic glycoprotein receptors. It is synthesized as a single polypeptide chain, which is subsequently cleaved into two disulfide-linked chains, HA1 and HA2 either during or after virus replication (Wilson et al., 1981). During the influenza virus replication cycle, the HA protein binds to a sialic acid containing receptor on the cell surface, bringing about the attachment of the virus particle to the cell (Klenk et al., 1955).

Amino acid changes in the HA can give rise to antigenic changes resulting in antigenic drift. Analysis of sequence data, suggests that both the number of amino acid substitutions and the location of amino acid substitutions can strongly influence the level of antigenic drift and the antigenicity of the virus (Daly et al., 1996). Study of the
HA1 of the human H3 influenza virus subtype resulted in the determination of five antigenic sites (A-E) that undergo sequence variation (Wiley and Skehel, 1987). These are shown in Table 1.2 and illustrated in Figure 1.2. As EI H3 viruses are thought to share a common ancestry with the human H3 subtype, these antigenic sites are believed to be universal in both species (Daniels et al., 1985). It was estimated that in humans a minimum of four amino acid changes in at least two of the five putative antigenic sites was necessary for antigenic drift to occur (Wilson and Cox, 1990). However, more recent data suggests that significant antigenic drift can result following changes to as few as one or two amino acids (Lewis et al., 2011; Jin et al., 2005). The HA gene is selected for phylogenetic analysis because it is the primary target against which neutralizing antibodies are generated and as a result, the evolutionary rate of the HA gene has been shown to be greater than other viral genes (Webster et al., 1992).

Table 1.2: Antigenic regions on the HA1 subunit protein

<table>
<thead>
<tr>
<th>Site A</th>
<th>Site B</th>
</tr>
</thead>
<tbody>
<tr>
<td>*121-126</td>
<td>132-137</td>
</tr>
<tr>
<td>*TAEGFT</td>
<td>QNGISG</td>
</tr>
<tr>
<td>*155-163</td>
<td>187-199</td>
</tr>
<tr>
<td>^TAEGFT</td>
<td>QNGISG</td>
</tr>
<tr>
<td>^TKSGNSYPT</td>
<td>SNQEQTKLYIQES</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site C</th>
<th>Site D</th>
</tr>
</thead>
<tbody>
<tr>
<td>*48-55</td>
<td>273-278</td>
</tr>
<tr>
<td>*MGKICNNS</td>
<td>PIDICV</td>
</tr>
<tr>
<td>^MGKICNNS</td>
<td>PIDICV</td>
</tr>
<tr>
<td>^RVTVSTKRSQTVIIPNG</td>
<td>DILMINSN</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site E</th>
</tr>
</thead>
<tbody>
<tr>
<td>*63-63</td>
</tr>
<tr>
<td>*RN</td>
</tr>
</tbody>
</table>

* = Amino acid sequence number; ^ = amino acid sequence

Adapted from Qi et al., 2010
Neuraminidase is the second largest glycoprotein on the influenza virus surface. This plays a key role in the release of replicating virus from the host cell and is also responsible for the breakdown of mucus on the upper respiratory tract allowing access of the virus to the epithelial cells. While the NA undergoes some degree of antigenic variation it is limited in comparison to antigenic drift observed in the HA gene. Influenza A viruses are divided into subtypes according to these two major surface
Chapter 1 Literature Review

glycoproteins (HA and NA). Wild aquatic birds are the natural reservoir of influenza A viruses and harbour all currently known 16 HA and 9 NA antigenic subtypes (Fouchier et al., 2005). Phylogenetic analysis suggests that viruses from aquatic birds were the ancestral source of all current lineages of mammalian viruses of which H1, H2, H3, N1 and N2 have circulated extensively in humans, H1, H3, N1 and N2 have circulated extensively in pigs and H3, H7, N7 and N8 have caused clinical disease in horses.

1.4 Epidemiology
Equine influenza virus (EIV) was first recognised in 1956 when a widespread epidemic of respiratory disease occurred in Eastern Europe (Sovinova et al., 1958). This virus (A/eq/1/Prague/56), which was a H7 haemagglutinin and a N7 neuraminidase, was designated as the prototype EIV, historically referred to as subtype 1. Outbreaks of respiratory disease caused by the H7N7 virus have decreased over time with the last confirmed outbreak being in 1979 (Webster, 1993). It has been reported however, that antibodies against this subtype 1 virus are sometimes still found in unvaccinated horses suggesting that it continues to circulate in a subclinical form at very low levels in some parts of the world (Madic et al., 1996).

In 1963 EIV of a different antigenic subtype (H3N8) caused a major epidemic in the state of Florida, USA (Waddell et al., 1963). This prototype subtype 2 virus (A/eq/Miami/63) was introduced into the USA horse population through the importation of horses from Argentina (Scholtens et al., 1964). The virus subsequently spread throughout the equine population of the Americas and Europe resulting in large outbreaks. In the late 1980’s and early 1990’s the H3N8 subtype diverged into two distinct evolutionary lineages designated European and American (Daly et al., 1996). These lineages were named initially based on the distribution of the viruses. However, as a consequence of increases in equine international travel, the geographical distribution of these viruses soon spread between continents. Both of these lineages continued to circulate independently. However, in Newmarket in 1993, viruses from American and European lineages were isolated from horses simultaneously in the same yard on the same day (Daly et al., 1996). It was suggested that viruses from the
American lineages may be more dominant however, as A/eq/Saskatoon/1/90 is the only “Eurasian” virus isolated in the western hemisphere to date (Oxburgh et al., 1998). However, a hypothesis by Koelle et al., (2010) carried out using a two-tiered model which can simulate both the ecological and the evolutionary dynamics of influenza viruses, suggests that the weakening of quarantine restrictions in Europe may have played a key role in shaping the evolutionary dynamics of EIV H3N8. HA analysis identified several amino acid changes in putative antigenic sites which are diagnostic of the division between American and European lineages (Daly et al., 1996) (Table 1.3). A study carried out on EI viruses from 1963 to 1994 estimated that the virus evolves at a rate of 2.5 nucleotide substitutions or 0.8 amino acid substitutions per year (Daly et al., 1996). More data involving EIV isolates from 1968 through 2007 suggest that the average rate of change was 2.1 nucleotide substitutions per year since and that the rate of change was approximately constant over time (Lewis et al., 2011).

Table 1.3: Amino acid substitutions “diagnostic” of the division between recent American and European isolates

<table>
<thead>
<tr>
<th>Location:</th>
<th>“European-like”</th>
<th>“American-like”</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78 163 189 207 213 273</td>
<td>261 276</td>
</tr>
<tr>
<td>Substitution:</td>
<td>V→D T→I N→K K→E I→V P→L R→K T→I</td>
<td></td>
</tr>
<tr>
<td>Site:</td>
<td>E B B D D near C near D near C</td>
<td></td>
</tr>
<tr>
<td>Strain:</td>
<td>Suf/89 Suf/89 Suf/89 Suf/89</td>
<td>Ken/91 Ken/91</td>
</tr>
<tr>
<td></td>
<td>Sx/89 Sx/89 Sx/89 Sx/89</td>
<td>Aru/91 Aru/91</td>
</tr>
<tr>
<td></td>
<td>Ell/89 Ell/89 Ell/89 Ell/89</td>
<td>Ken/92</td>
</tr>
<tr>
<td></td>
<td>Aru/91 Aru/91</td>
<td>N/1/93 N/1/93</td>
</tr>
<tr>
<td></td>
<td>Tab/91 Tab/91 Tab/91 Tab/91 Tab/91 Tab/91</td>
<td>Fl/93 Fl/93</td>
</tr>
<tr>
<td></td>
<td>Lam/92 Lam/92 Lam/92 Lam/92 Lam/92 Lam/92</td>
<td>Arg/93 Arg/93</td>
</tr>
<tr>
<td></td>
<td>H/K 92 H/K 92 H/K 92 H/K 92 H/K 92 H/K 92</td>
<td>Ken/94 Ken/94</td>
</tr>
<tr>
<td></td>
<td>N/2/93 N/2/93 N/2/93 N/2/93 N/2/93 N/2/93</td>
<td></td>
</tr>
</tbody>
</table>

Source: Adapted from Daly et al., (1996)
A later study of North American isolates indicated that the American lineage had further diverged into South American, Kentucky and Florida sublineages (Lai et al. 2001). Subsequent evolution within the Florida sublineage has resulted in the emergence of two distinct clades namely clade 1 and clade 2 (Figure 1.3). Clade 1 contains A/eq/Wisconsin/1/03 and A/eq/South Africa/4/03-like viruses which have been isolated predominantly in the USA, Japan and Australia (Bryant et al., 2009; Yamanaka et al., 2008, Callanan, 2008), while clade 2 contains the A/eq/Newmarket/5/03-like virus which has been isolated predominantly in Europe and associated with outbreaks in China and India (Bryant et al., 2009, Qi et al., 2010b, Virmani et al., 2010).
To date, EI has demonstrated almost worldwide circulation with the exception of a small number of island countries including New Zealand and Iceland. Bi-annual maps of worldwide EI activity recorded by the Office international des epizooties (OIE - world organisation for animal health) from July 2007 until December 2010 are illustrated in Figures 1.4 – 1.10. The maps of surveillance for this four year period were included in this thesis as it reflected the timeframe during which the majority of this research was conducted. At present EI is endemic in Europe and America (Bryant et
Countries such as Japan (Yamanaka et al., 2008), India (Uppal et al., 1989; Virmani et al., 2010) and South Africa (Guthrie et al., 1999; King and Macdonald, 2004) suffer occasional outbreaks. In 2007, Australia experienced its first incursion of EI following the importation of subclinically infected vaccinated horses (Callinan, 2008). Equine influenza has a low mortality rate but a very high morbidity rate resulting in major economic consequences in the event of disruption to equestrian events. Following the introduction of EI into South Africa in 1986, racing was suspended for 2.5 months costing a reported $70 million. In 2007, the outbreak of EI in Australia is reported to have resulted in financial losses close to one billion Australian dollars (Garner et al., 2011).

![Figure 1.4: Equine Influenza Activity (July – December 2007)](www.oie.int)

Source: OIE (www.oie.int)
Figure 1.5: Equine Influenza Activity (January - June 2008)
Source: OIE (www.oie.int)

Figure 1.6: Equine Influenza Activity (July - December 2008)
Source: OIE (www.oie.int)
Figure 1.7: Equine Influenza Activity (January - June 2009)
Source: OIE (www.oie.int)

Figure 1.8: Equine Influenza Activity (July - December 2009)
Source: OIE (www.oie.int)
Figure 1.9: Equine Influenza Activity (January - June 2010)
Source: OIE (www.oie.int)

Figure 1.10: Equine Influenza Activity (July -December 2010)
Source: OIE (www.oie.int)
Animals of all ages are susceptible to EI, however, the disease is particularly contagious in immunologically naïve populations, i.e., they have not been previously exposed by natural infection or received vaccination. In endemic countries where vaccination programmes are implemented, the disease is more commonly seen in young two and three year old performance horses (Newton et al., 2000). These horses are usually kept at high-density in poorly ventilated, shared airspaces at racetracks, training centres or sales facilities. Outbreaks of EI may occur at any time of the year, although seasonal outbreaks have been reported (Newton et al., 1999; Gildea et al., 2011).

1.5 Clinical signs and transmission

EI is characterised by typical clinical signs such as fever, harsh dry cough and nasal discharge (Van Maanen and Cullinane, 2002). Sporadic cases exhibiting neurological signs have also been reported (Daly et al., 2006a). The virus has a short incubation period of 1 to 5 days and this is proportional to the magnitude of the dose of the virus and the immunological status of the horse at the time of challenge (Cullinane, 2009; Gildea et al., 2011). Experimentally, it has been demonstrated that higher challenge doses shorten the incubation period, increase the duration of virus excretion and produce more severe clinical signs (Mumford et al., 1990). Longer incubation periods are presumably associated with a less challenging infective virus, which requires several rounds of replication within the horse before causing sufficient pathology for the horse to become clinically affected. Greatest viral shedding occurs in the early stages of clinical disease when coughing is most pronounced. Subclinically affected horses that may have been vaccinated or previously exposed to the virus can shed virus transiently without exhibiting clinical signs. These horses can be extremely difficult to recognise and pose a dangerous source of infection to naïve or partially protected horses (Daly et al., 2004a). The international transport of subclinically infected horses has been responsible for large outbreaks of EI, some of which occurred in South Africa, India, Hong Kong and Australia (Guthrie et al., 1999; King and MacDonald, 2004; Uppal et al., 1989; Powell et al, 1995; Garner et al, 2011).
EI is contracted during inhalation and the virus is transmitted primarily through the respiratory route. It has been reported that during coughing the virus can spread over distances of up to 32 meters and that in favourable air and wind conditions, the virus can spread over distances of up to 35 meters (Miller, 1965). Following the introduction of EIV into South Africa in 1986, the virus disseminated a distance of 1,600km following the release of two horses from quarantine (Guthrie, 1999) and during the Australian outbreak in 2007 it was reported that one index case travelled over 100 miles before transmitting the infection (Callinan, 2008). In addition to the aerosolised route, the virus can also travel indirectly by means of contact with contaminated fomites and can survive on fabrics, surfaces or equipment such as tack, grooming equipment and feeding utensils. At temperatures of 28ºC, influenza A viruses have been shown to survive on nonporous surfaces such as stainless steel and plastic for up to 48 hours and between 8-12 hours on cloth or paper (Bean et al., 1982). In an uncleaned horse transport vehicle, EIV has been demonstrated to survive overnight or for up to 12 hours (Guthrie et al., 1999). The virus can also be carried on skin and through human contact when failure to observe adequate hygienic precautions in handling and or transporting infected or non-infected animals occurs.

1.6 Pathogenesis

Following inhalation of the aerosolised virus, the majority of virus particles are deposited on the mucous film of the upper respiratory tract of the affected animal (Beech, 1991). Viral neuraminidase breaks down the mucosal layer (protective barrier over the cell surface) allowing access of the virus to the underlying epithelial cells (Matrosovich et al., 2004). The virus particles subsequently attach via their HA spikes to N-acetylneuraminic acid receptors on respiratory epithelial cells located in the upper respiratory tract (Figure 1.11). During replication, the virus enters the host cell by receptor-mediated endocytosis where the viral envelope fuses with an endosomal envelope. Uncoating of the influenza virions occurs, facilitated by the acidic pH of endosomes. Virus RNP subsequently enters the cell nucleus by active transport where all three types of viral RNAs: mRNA, cRNA, vRNA are synthesised (Shapiro & Krug, 1988). Viral HA, NA and M2 are transported through the golgi apparatus and become anchored in the plasma membrane (Zhang et al., 2000) where M1 assists in the
formation of virus particles. Thereafter, budding of progeny viruses occurs. Release from the host cell is mediated by the neuraminidase activity of NA, which destroys the sialic acid of the cellular and viral glycoproteins that would otherwise retain the new virions on the cell surface. Following release from the host cell, virus spreads quickly throughout the respiratory tract resulting in cell death, largely through apoptosis (Lin et al., 2002). Post infection, the muco-ciliary clearance mechanism can be severely impaired for up to 32 days (Willoughby et al., 1992). A recommended guideline is that horses should be stall rested for as many weeks as the number of days they suffered fever (Chamber et al., 1995). In a study carried out which examined the effects of exercising horses infected with influenza, results indicated that exercising horses that are acutely infected with influenza virus exacerbates the severity of clinical disease (Gross et al., 1998). Without rest and rehabilitation, opportunist bacteria can invade more easily and penetrate deeper giving rise to complications such as secondary bacterial infection.

![Figure 1.11: Illustration of Influenza A virus infecting host cell](http://publications.nigms.nih.gov/.../images/image3.png)
1.7 Diagnosis

In immunologically naïve horses EI is clinically recognisable, but in partially immune animals, laboratory tests may be required for a diagnosis. Diagnosis of EIV infection is achieved either by the detection of antigen or genetic material in clinical samples or by demonstrating an increase in the amount of antibodies against the virus in the horse’s blood using the haemagglutination inhibition (HI) or single radial haemolysis (SRH) test on acute and convalescent samples taken 10-14 days apart. Serology provides only retrospective data however, which does not facilitate prompt intervention in the event of an outbreak. Traditionally, HI tests have been used with detergent treated antigens in an effort to increase sensitivity of H3N8 antibody detection (John and Fulginiti, 1966).

A comparison of influenza serological techniques (HI/SRH) carried out in an international collaborative study involving seven laboratories demonstrated that the SRH test was the more sensitive and reproducible assay between laboratories (Mumford, 2000; Daly et al., 2007). As a result of these findings and the inherent variability reported by the HI test during the study, it was decided that the measurement of antibody levels in the present study would be carried out using the SRH test despite the fact that the HI test is more commonly used in diagnosis of EI as it is less labour intensive. A fourfold increase in HI titre or an increase of 25mm² or 50% of SRH antibody level is considered evidence of recent infection (OIE, 2008; Newton et al., 2000).

Isolation of virus from nasal pharyngeal swabs in embryonated eggs or Madin Darby canine kidney cells (MDCK cells) has traditionally been the gold standard of diagnostic tests for the detection of influenza virus. The chick embryo was first used for the isolation of the virus in 1935 (Smith, 1935) and to date, 8 to 12 day old embryonated hen’s eggs remain the primary method of EI propagation. Using this method, samples are collected from the nasopharyngeal area of horses exhibiting clinical signs of respiratory disease and injected into the allantoic cavity or amniotic sac of embryonated hen’s eggs (Figure 1.12). Virus is detected by haemagglutination (HA) activity within the amnion or allantois fluid of infected eggs and identity of the virus is then confirmed using specific antisera to H7N7 and H3N8 viruses in the HI test (OIE, 2008). Such techniques are labour intensive and may take up to several weeks when samples need to be passaged on numerous occasions (Daly et al., 2004a). In addition, the sensitivity of virus isolation is largely proportional to prompt sampling and processing of samples.
collected from suspect cases along with the presence and the amount of infectious material in the sample. During the 1989 epidemic in the UK, sensitivity of virus isolation in comparison with serology on paired serum samples was estimated at 50% (Livesay et al., 1993) and reports from the UK, Ireland and Sweden indicate that some EI viruses do not grow well in embryonated eggs rendering them difficult to isolate (OIE, 1996). Madin-Darby canine kidney (MDCK) cells can also be used for successful isolation of EIV however, it is considered that they are less permissive than embryonated eggs (Hannant and Mumford 1996) and may select for minor variants (Ilobi et al., 1994). Variants following passage in embryonated eggs have also been reported previously (Ilobi et al., 1998).

Figure 1.12: Steps in isolation of EIV in embryonated hens eggs

In recent years a wide variety of laboratory tests have become available that facilitate a more rapid diagnosis of EI in both clinical and subclinically affected animals by detecting the presence of viral antigens (Cook et al., 1988) or viral nucleic acid (Oxburgh and Hagstrom, 1999). A rapid immunoassay test kits that detects the presence of the nucleoprotein antigen of human influenza viruses within 15 minutes has been shown to be applicable to the diagnosis of influenza in the equine (Chambers et al., 1994). This is due to the degree of conservation in the nucleoproteins among influenza A viruses. Polymerase chain reaction (PCR) techniques enable the detection
of viral genetic material within 24-48 hours. This is a highly sensitive procedure and allows exponential amplification of short DNA sequences. There are two important advantages with the employment of this technique. Firstly, because PCR simply detects the presence of the viral genetic material, the method does not require live virus to be present in the sample material. Thus, if the virus has been destroyed following poor transportation conditions, stored inadequately or for a long period of time and subsequently cannot be isolated, PCR is still a viable option. Secondly, PCR of the HA gene directly from clinical samples combined with sequencing of the amplified products offers an additional advantage for genetic strain characterisation, which can yield valuable information for monitoring of antigenic drift (Ilobi et al., 1998). More recently, the advent of a second generation PCR method called real-time PCR, has introduced a quantitative aspect to this diagnostic technique. This technology relies on the detection and quantification of a fluorescent reporter. This is a signal, which increases, in direct proportion to the amount of PCR product in the reaction (Higuchi et al., 1993).

In a comparative study of diagnostic techniques to detect EIV, which included virus isolation, antigen detection (Directigen Flu-A) and reverse transcription-PCR (RT-PCR), the latter was found to be the most sensitive technique (Quinlivan et al., 2004). Quinlivan et al., (2005) later described the most sensitive method of detection for all type A influenza strains including EIV using real-time RT-PCR with a single set of primers designed by Fouchier et al., (2000) which amplifies a 244bp amplicon from nucleotides 32 to 276 of of the matrix gene. Despite these advances in EI diagnosis, virus isolation is the only diagnostic technique that yields a virus isolate that can be characterized antigenically and genetically. This is vital for surveillance of antigenic drift and provides virus for vaccine companies to update vaccine strains.
1.8 Prevention, management and control

Immunity

Protection against influenza virus relies largely on the production of antibodies against the virus HA and a definitive correlation between the level of these antibodies as measured by the SRH test and protective immunity against EI has been established in both experimental challenge studies and in the field (Mumford and Wood, 1992; Mumford et al., 1994a, Newton et al., 2000; Mumford 2001). Published data suggest that horses with antibody levels of 85mm$^2$ or greater are clinically protected (no clinical disease) against EIV and that those with antibody levels of 150mm$^2$ or greater are virologically protected (no clinical disease or virus shedding after challenge) (Mumford et al., 1994a, Newton et al., 2000; Mumford, 2001). Antibodies against the HA protein can occur following either natural infection or vaccination. However, the circumstances surrounding the induction of antibodies against EI may influence their correlation to protection. Resistance to re-infection has been reported to exist for one year and to persist even after levels of antibody become barely detectable (Hannant et al., 1988). The level of protection induced following vaccination or previous exposure is dependent on a close homology between the vaccine or previous exposure strain and challenge strain (absence of antigenic drift) as higher SRH antibody levels are required to protect against a heterologous virus (Newton et al., 1999; Daly et al., 2004b). The monitoring of antibody levels helps to determine when horses are most susceptible to influenza and when they benefit most from vaccination. An illustration indicating EI antibody levels (zones of haemolysis) as measured by the SRH test are shown in Figure 1.13.

![Figure 1.13: Zones of Haemolysis as measured by the SRH test](www.aht.org.uk)
**Vaccination**

Equine influenza vaccines provide protection against the virus by inducing antibody to the surface glycoproteins, in particular the HA. Mandatory vaccination of racehorses in the UK and Ireland was introduced in 1981 following a large-scale epidemic, which had affected racing two years earlier. After its introduction, EI was not diagnosed in either of these countries for almost a decade (Daly et al., 2006b). In the thirty years following the introduction of mandatory vaccination, no major equestrian event has been cancelled in Ireland as a result of EI. However, sporadic outbreaks and periodic disruptions to the training schedules of individual training yards have occurred. A current recommendation by the Turf Club advises that two initial primary injections be given 21-92 days apart followed by a booster injection 150-215 days later. Subsequent annual booster vaccinations should be administered thereafter (Turf Club, 2010).

Antigens for first generation vaccines are traditionally produced in embryonated eggs however methods of tissue culture preparations have also been developed. After propagation, virus is subsequently inactivated using either heat or chemical methods. As a result of their composition, these vaccines require a high antigenic mass and the inclusion of an adjuvant to induce an adequate immune response following vaccination (Mumford et al., 1994b). First generation vaccines include both traditional whole virus vaccines and more recent subunit vaccine preparations. Subunit vaccines contain purified HA and NA proteins and have been commercially available since the mid 1990’s. These vaccines contain new saponin adjuvants such as ISCOM’s or ISCOM-Matrix, and have been shown to stimulate significant levels of antibody against EIV (Mumford et al., 1994b). First generation vaccines commercially available in Ireland contain both subtypes of EIV i.e. H7N7 and H3N8 even though H7N7 no longer appears to be in circulation (Webster, 1993) and the OIE no longer recommend that a virus of this subtype be included in the vaccines (Cullinane et al., 2010). While the inclusion of the H7N7 subtype in vaccines is not longer a requirement, the measurement of antibodies against this subtype is a useful aid in the differentiation between vaccinated and naturally infected horses. The measurement of antibodies against the H7N7 virus has been carried out during the course of this study primarily for that reason. However, the recent development of a second generation canarypox vector vaccine (ProteqFlu-Merial) which contains representative strains of H3N8 only (Toulemonde et al., 2005) does not facilitate this method of differentiating between
vaccinated and natural infected horses. Nevertheless, during the Australian 2007 EI outbreak, where ProteqFlu was the vaccine of choice used to control and eradicate the disease, detection of antibody to the virus nucleoprotein by ELISA proved a useful aid in differentiating between those horses infected and those horses which had been vaccinated (DIVA) as antibodies against the virus nucleoprotein are only produced during natural infection (Sergeant et al., 2009).

This second generation or live vector EIV vaccine became commercially available in Ireland in 2005 and uses a canarypox vector to express the HA gene (Toulemonde et al., 2005). This vaccine is host restricted to certain avian species and produces an unproductive infection in mammalian cells (Plotkin et al., 1995). Live recombinant vectors vaccines aim to stimulate an immune response more closely resembling natural infection (stimulate both B and T cell responses) in comparison with inactivated vaccines, which require a high level of circulating antibody to provide clinical protection against the virus (Toulemonde et al., 2005). However their ability to do so is as yet largely unproven. In the USA and Canada a modified live intranasal influenza vaccine is also commercially available (Flu Avert IN, Heska Corp.) Safety and efficacy studies undertaken with Flu Avert IN have shown that this vaccine provides effective immunity for up to six months even in the absence of high levels of serum antibody (Townsend et al., 2001). The absence of sufficient humoral antibody levels for protection suggests that immune mechanisms other than humoral antibody are induced following vaccination.

**Efficacy of commercially available EI vaccines**

Despite the implementation of mandatory vaccination programmes, influenza outbreaks continue to occur and there has been an increased incidence in vaccinated horses since 2003 (Barquero et al., 2007; Gildea et al., 2011; Cullinane, 2009). This suggests limitations in the efficacy of some vaccines. A brief summary of some of the vaccine studies carried out to date suggested that an ISCOM vaccine (Equip, Pitman-Moore) conferred good protection post challenge for at least 15 months after three immunisations (Mumford et al., 1994b). An inactivated multivalent EI and EHV vaccine was demonstrated to induce significant antibody titres against EI using the SRH test following the second dose of primary vaccination (V2) however, these
antibody levels declined until the time of the third vaccination dose (V3). Post V3, antibody levels remained high for at least 12 months (Heldens et al., 2001). The efficacy of an updated multivalent EIV/EHV vaccine was examined by Heldens et al. (2004). Findings in this study suggested that vaccination protected against clinical signs and virus shedding 4 weeks after horses received their second vaccine dose i.e. at the time of optimum antibody levels. In addition, the SRH test demonstrated that vaccinated horses had high humoral antibody levels against EIV, above the required level for clinical protection 6 months after third vaccination dose (V3).

Edmund Toulemonde et al. (2005) investigated the efficacy of the second-generation canarypox recombinant vaccine in a small-scale experimental challenge study. Results indicated that the vaccine provided early protection in ponies challenged two weeks after immunisation with either 1 or 2 vaccine doses. It was concluded from this study that a single dose of ProteqFlu afforded protection in naïve animals in contrast to the two doses required by traditional inactivated vaccines. Two years later Minke et al., (2007) completed a follow-on study on the same vaccine which examined the duration of protective immunity provided following vaccination. Virological protection was demonstrated following challenge with a H3N8 virus two weeks after one vaccine dose; five months after two vaccine doses and 12 months after three vaccine doses. The greatest level of protection was observed two weeks following vaccination with one dose. This vaccine has also been demonstrated to stimulate cell-mediated immunity in addition to humoral immunity following challenge (Paillot et al., 2006). Alternative methods of protection arising from conventional inactivated vaccines e.g. cell-mediated immunity have yet to be demonstrated for many commercially available vaccine however an increase in the percentage of EIV-specific IFN-γ+ PBL has also been demonstrated following vaccination with an ISCOM based vaccine (Paillot et al., 2008).

More recently, a study by Heldens et al., (2009) examined the antibody response induced following vaccination with a novel ISCOM-Matrix vaccine using the HI test. This study demonstrated that the vaccine induced HI antibody levels that exceeded those required for clinical protection five months after the second dose of a primary course of vaccination. Clinical protection was also demonstrated following challenge and it was therefore suggested that this was the first inactivated EI vaccine that closes
the gap in immunity between the second (V2) and third (V3) dose of primary vaccination (Heldens et al., 2009). Similarly in 2010, Heldens and co-workers examined the duration of immunity induced by the same vaccine also using the HI test. The findings of this study suggested that the vaccine had the ability to induce HI antibody levels indicative of clinical protection 12 months post third vaccine dose (V3) (Heldens et al., 2010). While both of these studies indicate that this ISCOM-Matrix vaccine provides a good level of protection between second and third vaccine dose and up to 12 months following third vaccine dose, the use of the HI test to demonstrate these antibody levels indicative of protection is of questionable benefit. Similarly, differences observed in serological levels of protection demonstrated in studies carried out by Heldens and co-workers between 2001 and 2004 (HI) and 2009 and 2010 (SRH) may be more reflective in differences in the assays used to measure levels of EI protection rather than in qualitative differences in the vaccines.

Also in 2010, Paillot et al. (2010) carried out a study to examine the efficacy of a non-updated whole inactivated EI vaccine against the recent EIV isolate A/Sydney/2888-8/07 which was responsible for infected thousands of immunologically naïve horses in Australia. While nasal discharge was evident in both control and vaccinated ponies with no significant difference between the groups, the results of this study did indicate that this whole virus vaccine reduced clinical signs and virus shedding in vaccinates compared to control ponies two weeks after receiving their second vaccine dose (V2) i.e. at that time of optimum antibody levels.

While this data on individual vaccine products is useful, such experiments are generally undertaken under optimal conditions and a review of the literature identified a scarcity of comparative EI vaccine studies and conflicting results from several of those which had been performed. Mumford et al., (1994a) compared the immunogenicity of an ISCOM vaccine and an inactivated whole virus vaccine. Results of this study indicated that antibody responses were consistently higher and more durable in ponies vaccinated with ISCOMs than in those vaccinated with inactivated whole virus vaccine. This was contrary to the findings of Cullinane et al., (2001) who seven years later compared the immune responses elicited by a subunit ISCOM vaccine, an inactivated monovalent (containing EIV only) whole virus vaccine and an inactivated whole virus multivalent (containing EIV and EHV) vaccine and found that the monovalent whole virus vaccine
induced significantly better antibody responses than either the ISCOM or the multivalent vaccine. Subsequently a comparison of the antibody response induced following vaccination with a vaccine containing EI only versus an updated Immunostim multivalent EI/EHV vaccine was carried out and results suggested there was no significant difference in antibody responses of horses vaccinated with either of the two products (Heldens et al., 2002). More recently, the ability of two modern vaccines, a canarypox recombinant vaccine and an ISCOM-based vaccine to protect against a representative virus isolated from the Australian 2007 outbreak was evaluated. Bryant et al., (2010) reported that at two weeks post second vaccine dose (V2) i.e. at the time of peak antibody levels, the antibody response for both of these vaccines were similar. Such small-scale experimental efficacy studies do however have their limitations, as they involve only small numbers of experimental ponies and experimental conditions do not automatically equate to those in the field.

1.9 Surveillance and phylogenetic analysis

A fundamental part of influenza control programmes based on vaccination is active surveillance of the virus. Vaccines, that contain out of date strains are ineffective in controlling virus spread (Newton et al., 1999) and indirectly contribute to virus transmission through subclinically infected vaccinated horses (Daly et al., 2004a). The effect of out of date strains or antigenic drift on cross protection afforded by EI vaccines has been evaluated in hamster (Daly et al., 2003) and equine (Yates and Mumford, 2000; Daly et al., 2003) models. Results obtained in the latter study indicated that EI vaccines derived from American or European viruses, i.e. two distinct lineages, provided cross protection from clinical disease when animals were challenged with a European like virus. However, vaccines containing the European virus did not protect against the American strain when animals were challenged. Estimates of protective SRH antibody levels suggest that much higher levels are required for complete protection against a heterologous challenge as opposed to a homologous challenge (Mumford et al., 1988; Yates and Mumford, 2000; Daly et al., 2004b).

The effect of antigenic drift on EIV in the field was evident during outbreaks in the UK and Ireland in 1989 and in the UK in 1998 and 2003. During the 1989 outbreak, only horses with vaccine-induced antibodies of >200mm$^2$ were protected against infection.
This suggested that there had been significant antigenic changes from the virus circulating in the field compared to virus strains contained in the vaccines which had been isolated ten years earlier. In Ireland it was demonstrated that there were 16 amino acid differences between the HA of the field virus and the strains included in the vaccines (Nelly, 1996). Similar high levels of antibodies were required to prevent clinical disease during an outbreak of EI in the UK in 1998 (Newton et al., 1999). In Newmarket in 2003, many horses which had been vaccinated in the previous three months became clinically affected during an outbreak affecting 21 Thoroughbred training yards and more than 1300 racehorses (Newton et al., 2006.)

Antigenic characterisation of viruses isolated during this outbreak indicated that they were closely related to representatives of a sub-lineage of American viruses A/eq/Kentucky/5/02. This was the first reported isolation of this virus sub-lineage in the UK. Similar reports of antigenic differences between field and vaccine strains contributing to disease outbreaks were reported in Croatia 2004 (Barbic et al., 2009), in Italy in 2005 (Martella et al., 2007) and in Japan in 2007 (Yamanaka et al., 2008).

Such observations in the field are supported by data generated using mathematical modelling which also demonstrated that epidemics are more likely to occur when vaccines are not updated with circulating strains (Park et al., 2004). At present, only one of the five commercially available EI vaccines in Ireland has been updated in line with OIE recommendations of 2004 to contain a representative of the A/eq/South Africa/4/03 like virus and all vaccines need to be updated to include a A/eq/Richmond/07 like virus in line with the 2010 recommendations (OIE, 2010). The risk of EI infection due to vaccine breakdown is proportional to the antigenic distance between those viruses circulating in the field and those included in EI vaccines. Hence, there is a need for constant surveillance and antigenic characterisation of all EIV isolates where possible. Antigenic comparison of isolates can be performed using a panel of strain-specific reference antisera in a HI assay and genetic analysis is performed by analysis of the RNA sequence of the HA gene. Genetic analysis allows viruses to be placed in context with previous isolates and allows researchers to map the rate of antigenic change. In addition, genetic analysis also serves as a scientific basis for investigating the source of epizootics and outbreaks both nationally and internationally. This method of investigating the source of epizootics was previously employed during and EI outbreaks in South Africa in 2003 (King and MacDonald,
2004) and in Australia in 2007 (Callinan, 2008) and has proved valuable during the present study. Both antigenic and genetic analysis of isolates provides data for vaccine strain selection, which is carried out annually by the expert surveillance panel (ESP) appointed by the World Organisation for Animal Health (OIE). In humans, the formulation of vaccines is also reviewed on an annual basis however antigenic drift occurs at a higher rate in human influenza A viruses (Fitch et al., 1997) than that reported for EI viruses (Daly et al., 1996; Lewis et al., 2011; Murcia et al., 2011). Hence, EI vaccines do not have to be updated as frequently as human vaccines to be effective. The first formal recommendation by the ESP to update EI vaccine strains was made in 1993 and referred to the need to replace out of date strains from the late 1970’s and early 1980’s. Since then, only three further recommendations to update vaccine strains have been made (1995, 2004, and 2010). This rate of revising the H3N8 component of equine vaccines is in stark contrast with the H3N2 component of human influenza vaccines which has been updated almost 30 times since 1968 in order for the vaccine to remain effective (Russell et al., 2008).

In addition to the importance of updating vaccine strains, diligent international surveillance efforts are necessary to detect any new or emerging viruses e.g. A/eq/Jilin/89, previously seen in China. This virus resulted in a severe epidemic with unprecedented high morbidity and mortality in the Jilin and Heilongjiang Provinces in the northeast of the People’s Republic of China. In total approximately 20,000 horses were affected and the morbidity and mortality reached 81% and 20%, respectively in some herds (Guo et al., 1995). The virus responsible for the outbreak (A/eq/Jilin/89) was a H3N8 subtype virus; however, genetic analysis revealed it was unrelated to any H3N8 EI virus and more “avian-like” indicating that it had spread directly from the avian reservoir to horses (Guo et al., 1992). Although this avian-derived virus successfully transmitted to horses and lost its ability to replicate in ducks, it did not spread beyond China and did not persist in the local horse population after 1990 (Guo et al., 1995).

In Egypt, avian influenza virus H5N1 was identified during an outbreak of respiratory disease among donkeys in 2009 (Abdel-Moneim et al., 2010). This incident occurred one week after an outbreak of H5N1 infection in poultry in the surrounding area. H5 seroconversions by naturally exposed donkeys in the locality were also demonstrated (Abdel-Moneim et al., 2010). Both the outbreak of A/eq/Jilin/89 and
A/eq/Egypt/av1/09 emphasize the susceptibility of equine populations to avian influenza viruses. In the event that a whole avian influenza virus became infective for horses without losing its ability to replicate in birds, there would be a significant risk of global spread by aquatic birds during migration.

1.10 Interspecies transmission

A crucial feature of the ecology and epidemiology of influenza virus is interspecies transmission (Figure 1.14). Several factors must be present for interspecies transmission to occur, one of which is the presence of appropriate viral receptors on the respiratory tract in the newly established host to enable the HA to bind the virus to the host respiratory epithelial cells. The major sialic acid receptors in horses are alpha-2, 3 linked, similar to that in birds, whereas in humans the major sialic acid receptors are alpha-2, 6 linked (Weis et al., 1988; Connor et al., 1994; Gambaryan et al., 1995). The presence of appropriate viral receptors confers some species specificity and determines host range, in part (Baigent and McCauley 2003).

**Figure 1.14: The reservoir of influenza A viruses**

Source: [www.aht.org.uk/images/flu1.gif](http://www.aht.org.uk/images/flu1.gif)
In addition to the avian species being the natural reservoir, the pig may also act as an intermediate host for interspecies spread of influenza virus. The replication of all avian influenza viruses in this species supports this concept (Ludwig et al., 1995). In China, during 2004-2006 influenza virus surveillance isolated two strains of H3N8 influenza viruses from pigs. Sequence analysis revealed that the two swine isolates were of equine origin and similar to H3N8 European isolates from the early 1990’s (Tu et al., 2009). This would suggest that the horse may not be a dead end host for EI. Further evidence that the horse may not be a dead end host appeared in the spring of 2004 when the first reported evidence of influenza virus clinically affecting the canine species as a result of natural infection occurred (Crawford et al., 2005). Twenty-two racing greyhounds housed at a racing facility in Florida presented with clinical respiratory signs and high fever. Eight of the affected dogs died and influenza A virus was recovered from the lung tissue of one animal. Sequence analysis of the viral genome revealed a very close relationship with that of EI H3N8 of the Florida sublineage (Crawford et al., 2005). Four amino acid differences between the HA proteins in the equine and canine viruses were identified. It is probable that this resulted following the adaptation of the virus to a new host species. In the year that followed, influenza outbreaks occurred in numerous greyhound tracks and racing facilitaties in several states across the USA. By 2005 canine influenza (CI) had infected dogs in the general population and as of 2009, dogs from 30 states and the District of Columbia were positive for antibodies for CIV (Jirjis et al., 2010).

The interspecies transmission of EI to dogs was subsequently demonstrated not to be unique to the USA. Equine influenza was retrospectively shown to be responsible for an outbreak of respiratory disease in foxhounds in the UK in 2002 (Daly, 2006; Daly et al., 2008). The outbreak was flagged by a sudden onset of coughing in a pack of 92 hounds. One hound died and several were euthanized (Daly et al., 2008). Serum samples were collected from nine of the hounds affected by the respiratory disease between January and March 2005 and antibodies to A/eq/Newmarket/1/93 and A/eq/Newmarket/2/93 were detected. One of the nine hounds, which had detectable antibodies to equine H3N8 virus, was born in another location in the U.K. subsequent to the outbreak reported in 2002. This gives clear indication that the 2002 outbreak may not have been an isolated incident and that other outbreaks of equine H3N8 affecting foxhounds may have occurred (Daly et al., 2008). More recently, the transmission of
EI to dogs has also been reported during the recent outbreak of EI in Australia (Kirkland et al., 2010).

It is unclear what the precise association between horses and dogs is however, it is speculated that the interaction between the two may occur either via the respiratory route or through the consumption of infected equine carcasses by dogs and the latter could apply to both the greyhound and the foxhound population. The inhalation of avian influenza virus H5N1 by a dog through the consumption of raw lung material has previously been reported (Songserm et al., 2006). In an experimental challenge study the interspecies transmission of EI to dogs by close contact has been demonstrated (Yamanaka et al., 2009). Reports of canine influenza virus (CIV) in countries other than the UK, USA and Australia have not been documented however this may be due to a lack of surveillance. A phylogenetic tree illustrating the genetic relationship between CIV and EIV is shown in Figure 1.15.
1.11 Zoonotic potential of EIV

There is no evidence of EIV transmission to humans following exposure by natural infection in the field to date. However, because these viruses have already exhibited a change in species specificity by jumping from horses to dogs, this raises the question as to what other species may be susceptible to the H3N8 virus. In an experimental challenge study, mild influenza-like symptoms, virus shedding and seroconversion were observed in humans following challenge infection with A/eq/Miami/63 (Kasel et al., 1965). This demonstrated that the lack of relevant alpha-2, 3-linked receptor in humans is evidently not a complete barrier to EI infection. The increasing evidence of lack of species specificity is also supported by the fact that outbreaks of avian influenza H5NI have occurred in humans, predominantly in Asia, since 1997 (Claas et al., 1998).
In the event that EIV crosses the species barrier and adapts to humans it is possible that this could result in large outbreaks of clinical disease. Furthermore, should reassortment between EIV and human influenza A virus occur in humans this could potentially result in the development of novel virus strains to which there is no pre-existing immunity. In South America, results from a comparative serological study of horse sera using both equine influenza A viruses (H7N7 & H3N8) and human influenza A (H1N1 & H3N2) virus strains in the HI test indicated that sera from unvaccinated horses had higher mean antibody titres against human influenza strains in comparison to equine strains (Mancini et al., 2004). The results of this study also raised the question as to whether some horses may be exposed to human influenza virus, probably by their human handlers (Mancini et al., 2004).

1.12 Summary
Despite the introduction of mandatory vaccination in Ireland and the UK in 1981, EI outbreaks have continued to occur resulting in significant financial losses primarily due to disruption of training programmes, loss of performance and expenditure on veterinary assistance. While most EI outbreaks are sporadic and remain localised, the potential for large epizootics still exist, similar to that which occurred in Ireland and in the UK in 1989. In order to prevent such outbreaks and ensure business continuity in the equine industry, it is fundamental that we are competent in the control of EI. This necessitates in-depth surveillance, characterisation of circulating viruses and vaccination of highly susceptible/mobile populations with vaccines which contain epidemiologically relevant strains and are efficacious in providing both clinical and virological protection against EI.
1.13 References


Hirst, G. K. (1941) 'The Agglutination of Red Cells by Allantoic Fluid of Chick Embryos Infected with Influenza Virus', *Science*, 94(2427), 22-3.


King, E. L., Macdonald, D (2004) 'Report of the Board of Inquiry appointed by the Board of the National Horseracing Authority to conduct enquiry into the causes of the equine influenza which started in the Western cape in early December 2003 and spread to the Eastern Cape and Gauteng', Australian Equine Veterinarian, 23, 139-142.


Chapter 1 Literature Review


**Internet Sources:**

www.oie.int
www.ca.uky.edu
www.aht.org.uk
Chapter 2

Management and environmental factors involved in Equine Influenza outbreaks in Ireland from 2007 to 2010


Authors: Sarah Gildea, Sean Arkins, Ann Cullinane
2.1 Abstract

Reasons for performing the study: Outbreaks of equine influenza (EI) in endemic populations continue to cause economic loss despite widespread vaccination.

Hypothesis or Objective: The aims of this study were to identify the key management and environmental factors that determine the risk of horses contracting EI in an endemic country and to identify control strategies.

Methods: Real-time polymerase chain reaction (RT-PCR)/virus isolation was carried out on nasopharyngeal swabs and haemagglutination inhibition (HI) was carried out on clotted blood samples collected from horses and ponies showing signs of respiratory disease. On premises where a diagnosis of EI was confirmed, the attending veterinary surgeon was asked to participate in an epidemiological investigation.

Results: Between June 2007 and January 2010, EI outbreaks were diagnosed on 28 premises located in 13 of the 32 counties of Ireland. Veterinary advice was sought on average more than five days after the first clinical signs were observed. The majority of diagnoses were made by RT-PCR. Data from 404 horses on 16 premises were used in the epidemiological analysis. On 15 premises, EI was identified following the movement of horses. Housing type, teaser stallions or fomites/personnel contributed to virus spread. Vaccination status, number of year’s vaccination, time since last vaccination and age influenced disease expression. Isolation and vaccination were effective control measures on the premises where they were implemented.

Conclusions: Preventative measures include: isolation, clinical monitoring, serological testing and vaccination of new arrivals, booster vaccination of horses at six monthly intervals, maintenance of effective boundaries between equine premises and avoidance of stabling in single air spaces. Control measures include: prompt isolation of suspected cases, rapid diagnosis by RT-PCR, booster vaccination of cohorts and implementation of biosecurity measures to avoid transmission by fomites and personnel.

Potential relevance: Implementation of these preventative and control measures should reduce the economic losses associated with outbreaks of EI.
2.2 Introduction

Equine influenza (EI), which is caused by an Orthomyxovirus (equine influenza virus - EIV), is considered the most economically important infectious respiratory disease of horses (Timoney, 1996). This is due to the contagious nature of the virus and its potential to disrupt equestrian events. In susceptible horses the incubation period may be less than 24 hours, large quantities of virus are released into the atmosphere during coughing and the morbidity rate can be as high as 100% (Van Maanen and Cullinane, 2002). Disease spread can be explosive in immunologically-naïve populations. In Australia in 2007, after the first incursion of EIV, over 75,000 horses on over 10,000 properties became infected. A 72-hour nationwide “horse standstill” was imposed after which state-wide bans were maintained in New South Wales and Queensland prior to the introduction of zoning, vaccination and eventual eradication at an estimated cost of one billion Australian dollars (Garner et al., 2011). EI has been reported worldwide with the exception of a small number of island countries including New Zealand and Iceland. Countries such as Japan (Yamanaka et al., 2008), India (Uppal et al., 1989; Virmani et al., 2010) and South Africa (Guthrie et al., 1999; King and Macdonald, 2004) suffer occasional outbreaks and EI is endemic in Europe and America (Bryant et al., 2009).

In Europe and America the economic losses due to influenza are minimised by vaccination. Most major European Thoroughbred racing authorities have a mandatory vaccination policy. In the UK and Ireland racehorses are required to receive two primary vaccinations 21 to 92 days apart, followed by a third vaccination 150 to 215 days after the second and thereafter annual vaccination (Turf Club, 2010). Since 2005 the Federation Equestre Internationale (FEI) requires that all horses competing in their competitions have been vaccinated in the previous six months. Many horse and pony societies require vaccination and it is a prerequisite for entry in many Thoroughbred sales. Such policies serve primarily as insurance for business continuity and do not necessarily offer optimum protection. Outbreaks of EI continue to occur causing economic loss due to disruption to training programmes, loss of performance and expenditure on veterinary assistance. The standard recommendation is one week’s rest for every day of fever as the virus is pneumotropic and can cause damage to the ciliated respiratory epithelium. Regeneration of the respiratory epithelium and recovery of the
clearance mechanism may take weeks even in the absence of secondary bacterial infection (Willoughby et al., 1992).

This study describes an investigation of EI outbreaks in Ireland from June 2007 to January 2010. The aims were to identify the key management and environmental factors that determine the risk of horses contracting EI in an endemic country and to identify control strategies to minimise the impact of an outbreak.

2.3 Materials and Methods

2.3.1 Sample collection and clinical histories

Nasopharyngeal swabs and clotted blood samples were submitted to the Irish Equine Centre (IEC) by veterinary surgeons attending horses with clinical respiratory disease. After collection, the nasopharyngeal swabs were placed in 5 mls of virus transport medium (VTM) consisting of phosphate buffered saline (PBS), 5000u/ml penicillin, 250mg/ml amphotericin B and 2% v/v foetal bovine serum. On premises where a diagnosis of EI was confirmed, the attending veterinary surgeon was asked to participate in an epidemiological investigation. When the veterinary surgeon in consultation with their clients were in agreement to participate in this study, the author travelled to the premises for all subsequent samplings in an effort to obtain clinical/vaccination histories from the attending veterinary surgeon and personnel involved in the day-to-day management of the horses and to facilitate timely processing of samples. On the premises where EI was confirmed, morbidity was defined as the presence of one or more of the three most common clinical signs associated with influenza i.e. pyrexia, nasal discharge and coughing.

2.3.2 Real Time RT-PCR

RNA was extracted from 140µl of nasal secretions using the QIAamp Viral RNA Mini kit (Qiagen, Fleming Way, Crawley, West Sussex, UK) according to the manufacturer’s recommendations. One step RT-PCR was performed using Light Cycler RNA Amplification kit SYBR Green I (Roche Diagnostics, Charles Avenue, Burges Hill, UK) as previously described (Quinlivan et al., 2005).
2.3.3 Serological diagnosis
Sera were tested for antibodies against A/eq/Prague/56 (H7N7), A/eq/Kildare/89 (H3N8-European Lineage) and A/eq/Kildare/92 (H3N8-American Lineage) in 2007 using the haemagglutinin inhibition (HI) test. In 2008 the test was updated by replacing A/eq/Kildare/92 with A/eq/Meath/07. Sera were pre-treated with two volumes of potassium periodate and incubated at room temperature for 15 minutes. One volume of glycerol subsequently added and samples were incubated for a further 15 minutes at room temperature before heat inactivation at 56°C (+/- 1 °C) for 30 minutes. Testing was carried out in accordance with that described in the OIE manual (OIE, 2008). Seroconversion was defined as a fourfold or greater increase in antibody titre. Sera from confirmed cases of EI were also tested by single radial haemolysis (SRH). Antibodies against A/eq/Meath/07 (H3N8) a representative of the Florida sub-lineage of the American lineage and A/eq/Prague/56, the prototype H7N7 virus, were measured as previously described (Gildea et al., 2010). The results were expressed in mm² and a difference of 25mm² or greater between the H3N8 and H7N7 antibody levels was considered significant.

2.3.4 Virus isolation
RT-PCR positive samples (100 μl) were passaged up to four times in the allantoic cavities of 9 to 12 day-old embryonated hens’ eggs. Eggs were incubated at 34°C (+/- 1 °C) for 48 hours and then placed at 4°C (+/- 1°C) overnight before harvesting. The allantoic fluid was tested for haemagglutination activity (HA) using 1% chicken erythrocytes in PBS (OIE, 2008). Positive samples were confirmed by HI with type-specific ferret antisera supplied by the National Institute of Biological Standards (Potters bar, Hertfordshire, UK).

2.3.5 Statistical analysis
SPSS version 16.0 for Windows was used to analyse the data. Difference in age and number of years of vaccination history in horses that were clinically affected and their healthy cohorts was examined using an independent T test. Difference in duration of clinical signs between vaccinated and unvaccinated horses was also examined using this test. A Chi squared test was used to examine if there was an association between housing and clinical signs. Pearson’s correlation coefficient was used to examine if
there was a correlation between age and number of years of vaccination history and between age and H7N7 antibody titres. A significance level of $P < 0.05$ was used for all statistical tests.

### 2.4 Results

Between June 2007 and January 2010, EI was diagnosed on 28 premises located in 13 of the 32 counties of Ireland. These data are summarised in Table 2.1 and the geographical distribution of the affected counties is illustrated in Figure 2.1. Outbreaks occurred on a variety of premises among Thoroughbred horses, non-Thoroughbred horses and ponies (Table 2.1). The majority of diagnoses were made by RT-PCR. In total, 115 EIV RT-PCR positive nasopharyngeal swabs were submitted to the IEC from 2007 to 2010 from which virus was isolated from 12 (10.4%). Ten of the 12 (83.3%) viruses were isolated from swabs collected on the first day of sampling. Virus was detected over a maximum period of 15 days by RT-PCR on a Thoroughbred public stud farm (premises 16).

The nucleotide sequence of the haemagglutinin 1 (HA1) gene of six viruses isolated on different premises in 2007 was determined (data not shown). They belonged to clade 2 of the Florida sublineage of the American lineage. The analysis of limited sequence data generated for five additional viruses identified on other premises showed that they did not belong to clade 1 of the Florida sublineage. The sequencing of the HA1 gene of the virus responsible for the single outbreak in 2008 indicated that it also belonged to clade 2. All the eight viruses identified on five premises in 2009 belonged to clade 1 as did the three viruses identified on a single premises in 2010.

Following confirmation of EI by RT-PCR or serology, the attending veterinary surgeon was contacted, asked to provide information relating to the vaccination status of the horses and invited to participate in an epidemiological study. For 16 of the 28 premises the veterinary surgeon’s in consultation with their clients, agreed to participate in the study. The vaccination status of the horses on the 16 premises included in this study and of a further six premises where EI was diagnosed is summarised in Table 2.1. Data from 404 horses sampled on 16 premises were used in the epidemiological analysis and of these, at least 175 (43.3%) were described as clinically affected by the person
responsible for the day-to-day management of the horses. The following summarises
the findings on premises where investigations took place.

Table 2.1: EI in Ireland from 2007 to 2010; Premises number, date, location,
method of detection, type of premises and reported vaccination status of animals

<table>
<thead>
<tr>
<th>Premises Number</th>
<th>Date</th>
<th>Location (County)</th>
<th>Method of Detection</th>
<th>Type of Premises</th>
<th>Vaccination Status†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>June ‘07</td>
<td>Kilkenny</td>
<td>Serology</td>
<td>Private TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>2*</td>
<td>June ‘07</td>
<td>Fermanagh</td>
<td>Serology RT-PCR</td>
<td>Racing Yard</td>
<td>80.6%</td>
</tr>
<tr>
<td>3*</td>
<td>June ‘07</td>
<td>Clare</td>
<td>Serology RT-PCR</td>
<td>Show Jumping Yard</td>
<td>47.8%</td>
</tr>
<tr>
<td>4</td>
<td>June ‘07</td>
<td>Limerick</td>
<td>RT-PCR</td>
<td>Dealer’s Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>5*</td>
<td>June ‘07</td>
<td>Laois</td>
<td>Serology RT-PCR, VI</td>
<td>Polo Pony Yard</td>
<td>0%</td>
</tr>
<tr>
<td>6</td>
<td>July ‘07</td>
<td>Carlow</td>
<td>RT-PCR</td>
<td>Racing Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>7*</td>
<td>July ‘07</td>
<td>Donegal</td>
<td>Serology RT-PCR</td>
<td>Non-TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>8*</td>
<td>July ‘07</td>
<td>Donegal</td>
<td>Serology RT-PCR, VI</td>
<td>Non-TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>9*</td>
<td>July ‘07</td>
<td>Meath</td>
<td>Serology RT-PCR, VI</td>
<td>Racing Yard</td>
<td>89.3%</td>
</tr>
<tr>
<td>10</td>
<td>July ‘07</td>
<td>Kildare</td>
<td>RT-PCR</td>
<td>Racing Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>11*</td>
<td>July ‘07</td>
<td>Meath</td>
<td>Serology RT-PCR</td>
<td>Racing Yard</td>
<td>52%</td>
</tr>
<tr>
<td>12</td>
<td>Aug. ‘07</td>
<td>Carlow</td>
<td>RT-PCR, VI</td>
<td>Racing Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>13</td>
<td>Aug. ‘07</td>
<td>Carlow</td>
<td>RT-PCR, VI</td>
<td>Racing Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>14</td>
<td>Aug. ‘07</td>
<td>Kildare</td>
<td>RT-PCR, VI</td>
<td>Show Jumping Yard</td>
<td>Unknown</td>
</tr>
<tr>
<td>15*</td>
<td>Nov. ‘07</td>
<td>Mayo</td>
<td>RT-PCR</td>
<td>Mixed TB/Non-TB Public Stud</td>
<td>0%</td>
</tr>
<tr>
<td>16*</td>
<td>May ‘08</td>
<td>Down</td>
<td>Serology RT-PCR, VI</td>
<td>TB Public Stud</td>
<td>Described by V.S. as mixed,</td>
</tr>
<tr>
<td>No.</td>
<td>Date</td>
<td>Location</td>
<td>Test Method</td>
<td>Premises Type</td>
<td>Result</td>
</tr>
<tr>
<td>-----</td>
<td>------------</td>
<td>-----------</td>
<td>-------------</td>
<td>---------------</td>
<td>--------</td>
</tr>
<tr>
<td>17</td>
<td>Mar. ‘09</td>
<td>Kilkenny</td>
<td>RT-PCR</td>
<td>TB Public Stud</td>
<td>50%</td>
</tr>
<tr>
<td>18</td>
<td>Sept. ‘09</td>
<td>Galway</td>
<td>RT-PCR</td>
<td>Riding School</td>
<td>0%</td>
</tr>
<tr>
<td>19*</td>
<td>Nov. ‘09</td>
<td>Wexford</td>
<td>Serology RT-PCR</td>
<td>Hunting Yard</td>
<td>0%</td>
</tr>
<tr>
<td>20*</td>
<td>Dec. ‘09</td>
<td>Kilkenny</td>
<td>Serology RT-PCR</td>
<td>Racing Yard</td>
<td>73.9%</td>
</tr>
<tr>
<td>21*</td>
<td>Dec. ‘09</td>
<td>Donegal</td>
<td>Serology RT-PCR, VI</td>
<td>Riding School</td>
<td>0% (3 horses described as vaccinated by V.S.)</td>
</tr>
<tr>
<td>22</td>
<td>Dec. ‘09</td>
<td>Donegal</td>
<td>RT-PCR, VI</td>
<td>Non-TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>23</td>
<td>Dec. ‘09</td>
<td>Carlow</td>
<td>RT-PCR</td>
<td>Racing Yard</td>
<td>Described by V.S. as vaccinated</td>
</tr>
<tr>
<td>24*</td>
<td>Dec. ‘09</td>
<td>Donegal</td>
<td>Serology RT-PCR</td>
<td>Show Jumping Yard</td>
<td>0%</td>
</tr>
<tr>
<td>25</td>
<td>Dec. ‘09</td>
<td>Carlow</td>
<td>RT-PCR</td>
<td>Non-TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>26</td>
<td>Dec. ‘09</td>
<td>Clare</td>
<td>Serology RT-PCR</td>
<td>Pre-training TB Yard</td>
<td>Described by V.S. as mixed</td>
</tr>
<tr>
<td>27*</td>
<td>Jan. ‘10</td>
<td>Kildare</td>
<td>Serology RT-PCR</td>
<td>Non-TB Farm</td>
<td>0%</td>
</tr>
<tr>
<td>28*</td>
<td>Jan. ‘10</td>
<td>Limerick</td>
<td>Serology RT-PCR, VI</td>
<td>Racing Yard</td>
<td>43.5%</td>
</tr>
</tbody>
</table>

*= up to date vaccination records available; *Indicates premises where epidemiological investigations took place; TB = Thoroughbred; VI = virus isolation; V.S. = Veterinary Surgeon; Mixed vaccination status = some horses vaccinated and some unvaccinated; Mar. = March; Sept = September; Nov. = November; Dec. = December; Jan. = January.
Figure 2.1: Geographical Distribution (by county) of premises affected by EI from June 2007 to January 2010.

*Counties coloured yellow are those where EI was identified.

2.4.1 Spread between premises

In 15 of the 16 premises where investigations took place, EI was identified following movement of horses between premises. Horses on seven of the 15 premises were affected following attendance at various equestrian events including race meetings (premises 11, 20, 28) show jumping training shows (premises 3, 21, 24) and a local hunt (premises 19). The index cases on premises 21 and 24 had attended the same
training show. On six premises, horses were affected following the introduction of a new arrival of unknown vaccination status (premises 2, 5, 9, 15, 16, 27). On two premises clinical signs were observed on return of mares from public stud farms during the breeding season (premises 1, 7). Premises 8 was the only premises where there had been no reported movement on or off the premises for three months prior to the outbreak but this farm was contiguous to premises 7 with minimal boundary fencing.

### 2.4.2 Spread within premises

**Housing:** Accommodation details are presented in Table 2.2. Of the 283 horses for which this information was available, 42 were at grass, 142 were housed in stables, 83 were housed in barns and 16 horses were at grass during the day and housed in a barn at night. Eleven horses (26.2%) at grass, 59 (41.5%) in stables and 64 (77.1%) in barns were described as clinically affected. On premises where animals were kept at grass only, in stables only or in barns only 48.9%, 54.8% and 93.3% morbidity was observed respectively. On one premises (5) where animals were kept in a barn at night and at grass during the day, 50% morbidity was recorded.

Table 2.2: Premises number, vaccination status, housing type, morbidity and clinical signs on premises where epidemiological investigations took place

<table>
<thead>
<tr>
<th>Premises No.</th>
<th>Vaccination Status†</th>
<th>Housing Type (No. Horses)</th>
<th>Morbidity</th>
<th>Symptomatic/EI confirmed</th>
<th>Asymptomatic/EI confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0%</td>
<td>Grass (5)</td>
<td>1/5 (20%)</td>
<td>1/1</td>
<td>0/1</td>
</tr>
<tr>
<td>2*</td>
<td>80.6%</td>
<td>Mixed-barns (9), stables (7), grass (15)</td>
<td>13/31 (41.9%)</td>
<td>10/10</td>
<td>0/10</td>
</tr>
<tr>
<td>3</td>
<td>47.8%</td>
<td>Stables (17), grass (6)</td>
<td>4/23 (17.4%)</td>
<td>1/4</td>
<td>3/4</td>
</tr>
<tr>
<td>5</td>
<td>0%</td>
<td>Barn at night (16), grass during daytime (16)</td>
<td>8/16 (50%)</td>
<td>4/8</td>
<td>4/8</td>
</tr>
<tr>
<td></td>
<td>Grass (6)</td>
<td>Grass (10)</td>
<td>Stables (46), barns (10)</td>
<td>Stables (13), barns (9)</td>
<td>Barn (5)</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>-------------</td>
<td>--------------------------</td>
<td>--------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>7</td>
<td>0%</td>
<td>Grass (6)</td>
<td>4/6 (66.7%)</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>8</td>
<td>0%</td>
<td>Grass (10)</td>
<td>6/10 (60%)</td>
<td>2/3</td>
<td>1/3</td>
</tr>
<tr>
<td>9*</td>
<td>89.3%</td>
<td>Stables (46), barns (10)</td>
<td>16/56 (28.6%)</td>
<td>11/23</td>
<td>12/23</td>
</tr>
<tr>
<td>11</td>
<td>52.0%</td>
<td>Stables (25)</td>
<td>7/25 (28%)</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td>15</td>
<td>0%</td>
<td>Stables (13), barns (9)</td>
<td>4/22 (18.2%)</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>19</td>
<td>0%</td>
<td>Barn (5)</td>
<td>4/5 (80%)</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>20*</td>
<td>73.9%</td>
<td>Stables (23)</td>
<td>17/23 (73.9%)</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>21</td>
<td>0%</td>
<td>Barns (20)</td>
<td>20/20 (100%)</td>
<td>14/14</td>
<td>0/14</td>
</tr>
<tr>
<td>24</td>
<td>0%</td>
<td>Stables (8)</td>
<td>5/8 (62.5%)</td>
<td>4/4</td>
<td>0/4</td>
</tr>
<tr>
<td>27</td>
<td>0%</td>
<td>Barn (10)</td>
<td>10/10 (100%)</td>
<td>2/2</td>
<td>0/2</td>
</tr>
<tr>
<td>28</td>
<td>43.5%</td>
<td>Barns (20), stables (3)</td>
<td>23/23 (100%)</td>
<td>15/15</td>
<td>0/15</td>
</tr>
</tbody>
</table>

† = % up to date vaccination records available; *= Intermingling of horses at exercise areas (walker; gallops); Note: all the data were not available for premises 16 which was excluded from this table. The diagnosis of EI was confirmed by serological analysis and or by PCR based techniques.

**Fomites/personnel**: There was evidence to suggest that horses were infected following exposure to contaminated fomites or personnel on two premises (2, 24). On premises 2, a six-year-old gelding isolated in an American barn began exhibiting clinical signs six days after the index case. This horse was on box rest due to injury and did not have direct contact with any of the other horses in the yard. The horse tested positive by RT-PCR and seroconverted. On premises 24, a seronegative two-year-old gelding housed with a seronegative yearling exhibited clinical signs 10 days after the index case. These two horses were stabled full time and had no direct contact with any other horses in the yard. The yearling exhibited typical signs of influenza 24 hours after clinical signs were observed in the two-year-old. Both horses tested positive by RT-PCR and subsequently seroconverted.
Teasers: A teaser in the main yard of a large public stud (premises 16) exhibited clinical signs seven days after the arrival of the index case. A nasal swab collected from this animal two days later tested positive by RT-PCR. A second teaser on this premises also developed clinical signs three days later. Both teasers were isolated following the onset of clinical signs however frequent nose-to-nose contact with a large number of mares had already occurred. The resident veterinary surgeon reported that several of these mares subsequently developed clinical signs of influenza.

2.4.3 Case Study 1 - Premises 2
Clinical signs of nasal discharge, cough and lethargy were reported in a National Hunt training yard. Only partial sequence data, indicating that this virus did not belong to clade 1 of the Florida sublineage, was available for the virus identified on this premises. Examination of the passports indicated that 25 of the 31 horses (80.6%) had up-to-date vaccination records. The horses were vaccinated with vaccines that contained A/eq/Kentucky/94, A/eq/Newmarket/1/93 or A/eq/Kentucky/98 as a representative of the American lineage. There was a significant correlation between age and number of years of vaccination history (p <0.01). Sixteen horses were stabled in the yard and the remainder were at grass. Clinical signs were observed for between 6 and 17 days with a mean duration of 11.9 ± 1.02 SEM days overall and were only observed amongst the stabled horses (p <0.005).

The index case was a new arrival, a four-year-old of unknown vaccination history stabled in a row of loose boxes. Three days after arrival, the horse had a profuse nasal discharge and a persistent cough. Within the subsequent six days, a further 12 horses became clinically affected (Figure 2.2). The vaccination status of three clinically affected horses was unknown and all three had the same owner. The time since last vaccination of the other 10 clinically affected horses is summarized in Figure 2.2.

The pattern of virus spread on the premises is indicated in Figure 2.2. The index case was removed from its stable area five days after arrival i.e. 2 days after the onset of clinical signs and housed with two yearlings in a separate stable block. Both of these yearlings had received their first dose of EI vaccine two weeks prior to the outbreak. Within 24 hours, both yearlings exhibited clinical signs of EI and were reported as
being the most severely affected horses during the course of the outbreak. The last horse to develop clinical signs was injured and stabled alone (see above - fomites/personnel). Only clinically-affected horses tested RT-PCR positive.

### Figure 2.2: Pattern of EIV clinical signs on Premises 2

Coloured boxes = clinically affected; white boxes = not clinically affected; E = unoccupied box; Number in bold = onset of clinical signs (day); Index case (turquoise) = day 0 i.e. the first observation of clinical signs in the yard. Clinical signs for other EI infected horses were first observed on day +1 (purple), day +2 (green), day +3 (yellow), day +4 (orange), day +5 (pink), day +6 (blue), number in brackets = number of months since previous vaccination; U = Unknown vaccination record; * = received first dose of primary course only; \(^1\) = RT-PCR positive; \(^2\) = seroconversion; \(^3\) = RT-PCR positive and seroconverted to EIV.; \(\uparrow\) = movement of index case 2 days after the initial onset of clinical signs.

#### 2.4.4 Case Study 2 - Premise 9

Clinical signs of nasal discharge, coughing, lethargy and poor performance were observed in a Thoroughbred flat training yard. The virus identified belonged to clade 2 of the Florida sublineage. Examination of the passports indicated that 50 of the 56 horses (89.3%) had up-to-date vaccination records. All horses had been vaccinated with a vaccine containing A/eq/Newmarket/1/93 as the representative of the American lineage. There was a significant correlation between age and number of years of vaccination history (p < 0.01). There was also a significant association between age and
H7N7 antibody levels (p <0.01). All horses were stabled in the yard. Close contact between horses occurred daily within the confines of the walker and exercise facilities on this premises. Clinical signs were observed in 16 horses for between 3 and 24 days with a mean duration of 10.8 ± 1.37 SEM days overall. The pattern of disease spread on the premises and time since last vaccination is summarised in Figure 2.3.

The index case was a two-year-old colt, which arrived in the yard approximately 2 weeks prior to the outbreak. This horse had received its primary course of 3 doses of vaccine but had not received a booster dose in over 15 months. The index case was seronegative for H3N8 antibodies on initial testing but subsequently seroconverted. All five horses stabled adjacent to the index case subsequently developed clinical signs. One horse had received its first two doses of vaccine over 13 months previously and had never received its third dose. Ten additional horses in seven of the eight stabling areas developed clinical signs. The interval between the emergence of new cases was observed to be as short as 24 hours. Three horses were seronegative on initial sampling and were therefore fully susceptible to EIV in the event of an outbreak. Two of these had received booster vaccinations five months previously and the vaccination history of the third was unknown. In total, sampling was carried out on this premise on 5 occasions over a seven-week period. Of the 16 horses that were reported as clinically affected, 8 had RT-PCR positive nasal swabs and 7 seroconverted during the test period. Nasal swabs collected from six horses (10.7%) tested RT-PCR positive despite all six animals being reported as healthy during the outbreak (Figure 2.3). Five of the six horses had received booster vaccinations seven months previously while a 6th horse had received a booster vaccination five months previously. Similarly, seven other horses that seroconverted to EIV were also reported as clinically unaffected (Figure 2.3).
Figure 2.3: Pattern of EIV clinical signs on Premises 9

Coloured boxes = clinically affected; white boxes = not clinically affected; E = unoccupied box; Number in bold = onset of clinical signs (day); Index case (turquoise) = day 0 i.e. the first observation of clinical signs in the yard. Clinical signs for other EI infected horses were first observed on day +3 (purple), day +7 (red), day +8 (yellow), day +9 (orange), day +10 (pink), day +11 (blue), day +19 (green), number in brackets = number of months since previous vaccination; U = Unknown vaccination record; * = received first shot of primary course only; 1 = RT-PCR positive; 2 = seroconversion; 3 = RT-PCR positive and seroconverted to EIV.
2.4.5 Clinical signs

Veterinary advice was sought from 24 hours to 12 days (average 5.38 +/- 0.92 SEM days) after the first clinical signs were observed. The clinical signs most commonly described were persistent coughing and nasal discharge. No difference in pathogenicity was observed by the author on visiting affected premises and recording duration of clinical signs between outbreaks caused by clade 2 and those caused by clade 1 viruses. Where it was presumed that the index case was exposed to virus at an equestrian event, the average incubation period observed was 2.5 +/- 0.50 SEM days in horses from unvaccinated premises (19, 24), 3.0 +/- 0.41 SEM days in horses from mixed premises (3, 11, 21, 28) and 5 days in a horse from a premises (20) where up to date vaccination records were available for 73.9% of the horses. Table 2.3 indicates the average age of clinically-affected horses and their healthy cohorts. A significant difference was observed between the age of horses, which were clinically affected and their healthy cohorts on premises 2 (p <0.005), 9 (p <0.05) and 24 (p <0.05).

Table 2.3: Premises number, vaccination status and range of ages, average age of clinically affected and average age of healthy cohorts on 11 premises

<table>
<thead>
<tr>
<th>Premises No.</th>
<th>Vaccination Status† (range of ages-yrs)</th>
<th>Av. age (yrs) clinically affected +/-SEM</th>
<th>Av. age (yrs) healthy +/-SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>2*</td>
<td>80.6% (1-13)</td>
<td>4.1 +/- 0.63</td>
<td>7.8 +/- 0.67</td>
</tr>
<tr>
<td>3</td>
<td>47.8% (1-15)</td>
<td>4.8 +/- 0.75</td>
<td>6.8 +/- 1.15</td>
</tr>
<tr>
<td>5</td>
<td>0% (5-15)</td>
<td>7.1 +/- 0.44</td>
<td>8.75 +/- 1.05</td>
</tr>
<tr>
<td>7</td>
<td>0% (&lt;1-6)</td>
<td>3.0 +/- 1.23</td>
<td>2.0 +/- 0.00</td>
</tr>
<tr>
<td>8</td>
<td>0% (&lt;1-8)</td>
<td>4.8 +/- 1.14</td>
<td>4.5 +/- 1.71</td>
</tr>
<tr>
<td>9*</td>
<td>89.3% (2-7)</td>
<td>2.3 +/- 0.12</td>
<td>3.2 +/- 0.22</td>
</tr>
<tr>
<td>11</td>
<td>52.0% (2-9)</td>
<td>3.0 +/- 0.63</td>
<td>5.0 +/- 1.34</td>
</tr>
<tr>
<td>15</td>
<td>0% (&lt;1-18)</td>
<td>4.6 +/- 2.40</td>
<td>7.2 +/- 1.59</td>
</tr>
<tr>
<td>19</td>
<td>0% (&lt;1-18)</td>
<td>5.5 +/- 2.22</td>
<td>18.0 +/- 0.00</td>
</tr>
<tr>
<td>20</td>
<td>73.9% (2-9)</td>
<td>5.4 +/- 0.51</td>
<td>5.8 +/- 0.31</td>
</tr>
<tr>
<td>24*</td>
<td>0% (1-12)</td>
<td>2.4 +/- 0.40</td>
<td>8.3 +/- 2.03</td>
</tr>
</tbody>
</table>

† = % up to date vaccination records available; * = Significant difference; Av = average; yrs = years
2.4.6 Serological status

The SRH results for first serum sample collected from the 118 EI confirmed cases on the premises investigated are summarised in Table 2.4. Thirty one horses (26.3%) were seronegative for H7N7 and H3N8 on initial sampling. Of these, only four had up-to-date vaccination records and 23 were described as unvaccinated. All 31 horses were described as clinically affected and two were identified as the index case on affected premises. Fifty three horses (44.9%) were seronegative for H7N7 but seropositive for H3N8 at the time of sampling. Of these, 10 had up-to-date vaccination records and 29 were described as unvaccinated.

Seventeen horses (14.4%) had H3N8 SRH levels that were at least 25mm$^2$ higher than their H7N7 SRH levels. Of these, 10 had up to date vaccination records but two had received their first dose of vaccine only two weeks prior to the outbreak. Seventeen horses (14.4%) had comparable SRH levels for H7N7 and H3N8. Of these, nine had up to date vaccination records and the vaccination status of the remaining eight was unknown. All 17 horses had SRH antibody levels $\geq 85$mm$^2$ against H3N8 i.e. indicative of clinical protection and six of them had SRH antibody levels of $\geq 150$mm$^2$ i.e. indicative of virological protection at the time of initial sampling.

Table 2.4: SRH data for confirmed cases of EI on initial sampling

<table>
<thead>
<tr>
<th>Premises ID</th>
<th>Number of confirmed cases</th>
<th>No. seronegative H7N7 and H3N8</th>
<th>No. seronegative H7N7&gt; H7N7 seropositive H3N8</th>
<th>No. seropositive H3N8&gt; H7N7</th>
<th>No. seropositive H3N8=H7N7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>0</td>
<td>7</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>3</td>
<td>6</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>20</td>
<td>6</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td>19</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
2.4.7 Incidence of disease among vaccinated and non-vaccinated horses

Of 34 horses with up-to-date vaccination records that were described as clinically affected, 21 (62%) had not received a booster vaccination in the previous six months. Of the 13 horses that had been vaccinated in the previous six months two horses had only received their first vaccine dose two weeks prior to the outbreak. On premises 2 (case study 1) there was a significant difference (P<0.05) in the number of years’ vaccination history of those that were clinically affected (1.6 yrs ± .09 SEM) compared to their healthy cohorts (4.5 yrs ± .60 SEM).

On premises 21, which had a mixed vaccination status, it was reported by the owner of the premises that three vaccinated livery horses exhibited less severe clinical signs than their 17 unvaccinated cohorts. The average duration of clinical signs exhibited by the vaccinated livery horses was 7.7 +/- 0.67 SEM days, which was significantly less than the 14.4 +/- 0.40 SEM days in their unvaccinated cohorts (p <0.001). Initial sampling took place nine days after the first clinical signs were observed in two of the livery horses. The third livery horse only exhibited very mild signs of respiratory disease and was sampled eight days later. All three livery horses tested RT-PCR negative but 11 of their cohorts were RT-PCR positive. The livery horses were seropositive for both H7N7 and H3N8 viruses on initial sampling, consistent with their vaccination history. Of the 17 cohorts described as unvaccinated, one had detectable antibodies against H7N7 on initial sampling. Four were seronegative for both H7N7 and H3N8 viruses consistent with their unvaccinated status. The remaining 12 horses had antibodies against H3N8 but not against H7N7 suggesting recent exposure to virus by natural infection.

<table>
<thead>
<tr>
<th></th>
<th>21</th>
<th>14</th>
<th>6</th>
<th>8</th>
<th>0</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>15</td>
<td>8</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>118</td>
<td>31 (26.3%)</td>
<td>53 (44.9%)</td>
<td>17 (14.4%)</td>
<td>17 (14.4%)</td>
<td></td>
</tr>
</tbody>
</table>

Clinically affected cases = EI confirmed by RT-PCR or serology; No. = number; H3N8 > H7N7 = H3N8 antibody level of ≥ 25mm² more than H7N7 antibody level. H3N8 = H7N7, H3N8 antibody level not ≥ 25mm² more than H7N7 antibody level.
2.4.8 Control

Isolation/Biosecurity: Isolation protocols were implemented following the onset of clinical signs on premises 1, 3 and to a limited extent on premises 16. In addition, strict biosecurity measures such as disinfection and changing of clothing and equipment were implemented on premises 1 and 16. The index case on premises 1 returned home from a non-Thoroughbred public stud showing typical clinical signs of EIV. This mare was isolated immediately on arrival and biosecurity measures were implemented by her owner, a veterinary nurse. There was no evidence of disease spread to her unvaccinated cohorts. On premises 3, the index case was isolated within 24 hours of becoming clinically affected and disease spread was limited to three other horses (17%) on the farm. The index case on premises 16, a large Thoroughbred public stud farm, began to show clinical signs of EI less than 12 hours after being admitted to the arrivals barn. This mare and her foal were immediately placed in isolation. However, they had been in contact with 10 other mares, which were subsequently dispersed to other yards on the stud. The index case and four of these mares tested positive by RT-PCR. During the course of the outbreak, the resident veterinary surgeon ensured that movement of horses between yards was restricted, mixing of visiting mares and resident mares was prohibited, and examination/treatment of clinically affected horses was only carried out after the examination/treatment of healthy horses. A further 18 nasal swabs were collected of which six (33.3%) were RT-PCR positive. No evidence of virus spread from this stud farm to other premises was reported.

Vaccination in the face of an outbreak: On premises 2 (case study 1) booster vaccinations of Equilis Resequin (Intervet, Boghall Road, Bray, Co Wicklow, Ireland) were given to all healthy horses 10 days after the first clinical signs were observed. No new cases were observed post vaccination. On premises 3 all horses were vaccinated with Equilis Equenza, (Intervet) in the face of the outbreak. No new cases were observed post vaccination.
2.5 Discussion

EI was diagnosed on 28 premises in 13 counties in Ireland between June 2007 and January 2010. The location and incident of confirmed cases may, to some extent, be biased by the level of interest of the attending veterinarian in obtaining a laboratory diagnosis. The diagnosis of EI on the majority of premises (27 of the 28) was made by RT-PCR. This technique has previously proved to be more sensitive than virus isolation, antigen detection by the Directigen Flu A kit or serology, and to be effective in the detection of virus shedding from seropositive horses (Quinlivan et al., 2004; 2005). In this study horses on all the premises where EI vaccination was routinely carried out, tested positive by RT-PCR including six subclinically infected horses. The role of subclinically infected vaccinated horses in the international spread of EIV has been highlighted in recent years and RT-PCR has been introduced as a routine screening procedure for imported horses in Australia (Cullinane, 2009).

Virus was isolated from only 10.4% of the RT-PCR positive nasal swabs identified during this study. All but two of these nasal swabs were collected on the first day of sampling. It is likely that this low rate of isolation was due to a delay in veterinary intervention and therefore peak viral shedding being missed in sampling. Veterinary advice was sought on average more than five days after the first clinical signs were observed. Delayed veterinary intervention results in an over reliance on RT-PCR as a diagnostic tool and a delay in the implementation of appropriate control procedures. The timely collection of nasopharyngeal swabs is necessary for virus isolation and the monitoring of viruses for antigenic drift. The latter is essential for influenza surveillance and the updating of vaccines. The delay in veterinary intervention also affected the serological analysis in this study as many of the horses had already mounted an antibody response to virus challenge at the time of initial sampling. This led to difficulties in determining an association between EI infection and the level of antibodies at the time of virus exposure.

During the investigations, inadequate vaccination, movement, contact, age and housing were identified as potential risk factors in the spread of EI. Only four of the 28 affected premises were described as vaccinated by the attending veterinary surgeon. On six premises, clinical signs were observed following the introduction of new horses with unknown or out of date vaccination records. The risk associated with failing to isolate
new arrivals and establish their antibody status was particularly well illustrated in two racing yards (case studies 1 and 2) where the majority of horses had up to date vaccination histories but the final morbidity rate in both cases was estimated to be over 28%.

2.5.1 Risk Factors

Virus Spread

With the exception of premises 7 and 8, which were contiguous, the outbreaks were limited to single premises. There was no evidence that frequent movement of horses from some of the 16 infected premises identified in this study led to the widespread dissemination of virus and the occurrence of outbreaks on other premises. A recent study where the protection against EIV was predicted from serum antibody titres suggested that 83% of Thoroughbreds in training and 51% of non-Thoroughbred horses tested in Ireland were clinically protected (Gildea et al., 2010). Virus spread in the partially immune Irish horse population during from 2007 until 2010 was in stark contrast to that observed in 2007 in the immunologically naïve population in Australia but similar to that observed during the 2003 outbreak in Newmarket. In Australia the participation of an infected horse(s) at a “one day event” resulted in the spread of the virus to more than 50 different premises (Callinan, 2008). Subsequently, a study was carried out to quantitatively analyse the risk of spread E1 following movements of vaccinated horses from infected areas during the Australian outbreak. This results of this study indicated that testing horses twice during pre-movement isolation and again during post-movement isolation should minimise the likelihood of virus spread (Sergeant et al., 2011). Very limited virus spread was observed through the racing network during the 2003 outbreak in Newmarket; it was suggested that only healthy, and consequently less infectious, horses would have been likely to attend race meetings (Newton et al., 2006).

Inadequate Vaccination

Initial serological analysis of the index cases on all premises investigated indicated that they were unvaccinated, inadequately vaccinated or had responded poorly to vaccination (data not shown). On premises 21, which had a mixed vaccination status, clinical signs were much reduced in the vaccinated horses. On premises 2 there was a significant relationship between the number of years of vaccination history and the
absence of clinical signs. On vaccinated premises 2, 9 and 20, in excess of 75% of horses that were clinically affected had not received a booster vaccination in the six months prior to the outbreaks. This concurs with the findings of Barquero et al., (2007) where horses that had not received a booster vaccination in the three months prior to the 2003 outbreak in Newmarket were at greater risk of infection.

A definitive correlation between antibody levels against the virus haemagglutinin protein, as measured by the SRH test and protective immunity against EI has been established. Experimental studies and observations in the field suggest that horses with antibody levels of 85mm$^2$ or greater are clinically protected against EIV and that those with antibody levels of 150mm$^2$ or greater are virologically protected and do not shed virus after challenge (Mumford et al., 1994; Newton et al., 2000; Mumford, 2001). It is not possible to differentiate between H3N8 antibodies due to natural exposure and those due to vaccination, but the measurement of antibodies against H7N7 provides information concerning horses vaccinated with all but one of the EI vaccines commercially available on the Irish market, as they contain a representative of that subtype. In vaccinated horses the SRH antibody levels against H7N7 and H3N8 usually correlate and a higher H3N8 level suggests exposure by natural infection (Gildea et al., 2010).

In this study over 70% (84 of the 118 horses) with EI (as confirmed by serology or RT-PCR) were seronegative for H7N7 suggesting that they had no vaccinal antibodies at the time of exposure. Of these 84 horses, 52 were described as unvaccinated but 12 had up-to-date records of vaccination (with a vaccine containing an H7N7 virus) suggesting that they had responded poorly to vaccination. Their average time since last vaccination was > 7 months. On initial sampling 17 of the 118 horses with EI had similar antibody levels for H3N8 and H7N7 suggesting that their H3N8 antibodies were vaccinal and that they had not mounted an antibody response to virus exposure at the time of sampling. All 17 had SRH levels ≥85mm$^2$ against H3N8 and six of them had antibody levels ≥150mm$^2$. Thus, these horses had responded well to vaccination but the majority were inadequately protected as 11 of the 17 (65%) were clinically affected. Vaccination records were available for nine of these horses, three of which were described as clinically affected. All nine had been vaccinated with vaccines that had not been
updated in accordance with the OIE recommendations of 2004 i.e. the vaccines did not contain a representative of the Florida sublineage. Vaccine mismatch reduces protection against infection and virus shedding and higher levels of antibody are required to protect horses against heterologous strains (Newton et al., 1999).

Movement
Periods of increased risk associated with seasonal increased mixing patterns among horses have previously been identified by Newton et al., (1999). In this study EI was diagnosed on 15 of the premises following the movement of horses between different populations predominantly in the summer and winter seasons. The only premises (8) where there was no movement was contiguous to an affected premises (7) and the horses had nose-to-nose contact across a boundary fence. Close contact between teasers and broodmares on premises 16 similarly assisted virus spread. A study by Morley et al., (2000) identified frequent direct contact between exercise ponies and racehorses as being strongly associated with the risk of influenza.

Age
In an endemic country older horses older horses may have had prior exposure to EI as a result of natural infection and if vaccinated are likely to have a longer history of vaccination than their younger cohorts. On three premises (2, 9, 24) a significant difference was observed between the incidence of clinical signs among younger and older horses (i.e. young horses had a greater risk of influenza than their older cohorts). A significant association between age and number of years of vaccination was established on premises 2 and 9. The horses on premises 24 were described as unvaccinated and no history of previous exposure by natural infection was available. The delay in veterinary intervention and sampling on these premises made it impossible to determine the H3N8 antibody level prior to virus exposure. The findings that younger horses were more susceptible to EI are consistent with those of Morley et al., (2000) from a three-year study in Canada where influenza is endemic and vaccination is widely practiced. In the 2003 Newmarket outbreak there was evidence of increased risk of infection in older horses, however this was explained by more recent vaccination among younger horses (Barquero et al., 2007).
Chapter 2 Experimental

Housing
On investigation of housing types, in excess of 77% of horses stabled in barns, 41% of horses in stables and 26% of horses at grass became clinically affected. It should be noted that there was considerable variation in infection rates between different premises with the same housing type. In this study it was not possible to monitor proximity, the extent of interaction between individuals and the amount or duration of virus shedding, all of which may have impacted on virus transmission. However, the study suggests that horses stabled in collective air spaces may be at greater risk of EI. This is consistent with the findings of Morley et al., (2000) during the 1992 epidemic in Saskatoon, Canada, when horses stabled in shed barns were found to be approximately four times more likely to develop respiratory signs that those housed in pole barns with better ventilation. Similarly it was suggested that high density stabling with a common air space facilitated the transmission of virus during the 1992 outbreak of EI in Hong Kong (Powell et al., 1995). In addition to housing, contaminated fomites or personnel assisted in virus spread on two premises during this study. Following the introduction of EI into South Africa for the first time in 1986, contaminated fomites played a key role in subsequent virus spread (Guthrie et al., 1999). Contaminated personnel or equipment have also been implicated in the spread of disease during a second outbreak in South Africa in 2003 and during the Australian outbreak in 2007 (King and MacDonald, 2004; Callinan, 2008).

Control
During the 2007-2010 outbreaks in Ireland biosecurity measures, isolation, and vaccination were employed to reduce the impact of EI. Studies have proven that influenza A virus can survive for between 24 and 48 hours on hard surfaces (Bean et al., 1982) and that EIV can survive in tap water for 14 days at 4°C (Yadav et al., 1993). Contaminated vehicles, fomites and personnel have been implicated in the spread of EI (Guthrie et al., 1999; King and Macdonald, 2004; Callinan, 2008). In this study, it was concluded that in the absence of contact with infected horses, the virus was spread by personnel or fomites on two premises, emphasising the need to implement biosecurity measures in the face of an EI outbreak. On two of the three racing yards investigated and on one Thoroughbred stud farm, extensive disinfection was carried out to reduce virus spread by personnel and contaminated fomites. The stud farm also isolated the
index cases within 24 hours of the onset of clinical signs and appeared to be successful in limiting the outbreak, as no spread to secondary premises was reported. The effectiveness of prompt isolation was illustrated on premises 1 where the infected mare was quarantined and there was no evidence of virus spread. Furthermore, results from this study are consistent with the findings of Barquero et al., (2007) who also support vaccination in the face of an outbreak. Similarly, a study carried out by Baguelin et al., (2010) using a metapopulation model also demonstrated the likely benefits of trainers revaccinating their horses following local diagnosis of influenza. Moreover, such benefit were greatly enhanced when a significant number of other trainers revaccinated. During recent EI outbreaks South Africa (2003) and in Australia in (2007) vaccination in the face of the outbreak minimised virus spread and assisted in completing eradicating the virus from both (Guthrie et al., 1999; Garner et al., 2011). In this study vaccination was implemented on premises 2 and 3 soon after initial diagnosis and no new cases were identified.

Conclusion and Recommendations
In conclusion, the findings of this study provide a foundation for targeted preventative and control measures for EI in an endemic situation. Preventative measures recommended as a result of these findings include: (1) isolation and clinical monitoring of new arrivals and horses returning from equestrian events, (2) serological testing of new arrivals and vaccination as appropriate, (3) vaccination of horses, especially young horses and teasers at six monthly intervals, (4) maintenance of effective boundaries between equine premises and (5) avoidance of stabling in single air spaces. Control measures recommended as a result of these findings include: (1) prompt isolation of suspected cases, (2) rapid confirmatory diagnosis by RT-PCR, (3) booster vaccination of cohorts following early diagnosis and (4) implementation of strict biosecurity measures to avoid transmission by fomites, personnel and contaminated vehicles.
Acknowledgements
We would like to express our gratitude to all the veterinary clinicians, trainers and horse owners who cooperated with this study. They were extremely generous with their time and forthcoming with information for the benefit of the equine industry. We would also like to thank Dr. Wayne Martin for helpful discussions. The assistance of the staff in the virology unit with the diagnostic testing during the outbreaks is gratefully acknowledged. The epidemiological investigations and experimental work was funded by the Department of Agriculture under the National Development Plan. All of the experimental work was carried out at the Irish Equine Centre. The results will be submitted as part of a PhD thesis by Sarah Gildea to the University of Limerick.

2.6 References


King, E. L., Macdonald, D (2004) 'Report of the Board of Inquiry appointed by the Board of the National Horseracing Authority to conduct enquiry into the causes of the equine influenza which started in the Western cape in early December 2003 and spread to the Eastern Cape and Gauteng', *Aus Equine Vet*, 23, 139-142.


Chapter 3

The molecular epidemiology of equine influenza in Ireland from 2007-2010 and its international significance

Published in: Accepted for publication by: Equine Veterinary Journal

Authors: Sarah Gildea, Michelle Quinlivan, Sean Arkins, Ann Cullinane
3.1 Abstract

**Reasons for performing the study:** Antigenic and genetic drift of equine influenza (EI) virus is monitored annually by the Expert Surveillance Panel (ESP), which make recommendations on the need to update vaccines. Surveillance programmes are essential for this process to operate effectively and to decrease the risk of disease spread through the international movement of subclinically infected vaccinated horses. Not only is surveillance necessary to inform vaccine companies which strains are in circulation, but it serves as an early warning system for horse owners, trainers and veterinary clinicians, facilitating the implementation of appropriate prophylactic and control measures.

**Objective:** To summarise the genetic analysis of EI viruses detected in Ireland between June 2007 and January 2010.

**Methods:** The HA1 genes of 18 viruses were sequenced and phylogenetic analysis undertaken.

**Results:** All viruses belonged to the Florida sublineage of the American lineage. Clade 2 viruses predominated up to 2009. The viruses identified on four premises in 2007 displayed 100% nucleotide identity to A/equine/Richmond/1/07 (H3N8), the current clade 2 prototype. The first clade 1 virus was identified in November 2009 and, thereafter, clade 1 viruses were responsible for all the outbreaks identified. The Irish clade 1 viruses differ from the clade 1 virus responsible for the EI outbreaks in Japan and Australia in 2007. No virus of the Eurasian lineage was isolated during this surveillance period.

**Conclusions:** In 2010 the ESP recommended that the vaccines should not include a H7N7 virus or a H3N8 virus of the Eurasian lineage but that they should contain both a clade 1 and clade 2 virus of the Florida sublineage. The surveillance data presented here support these recommendations and indicate that they are epidemiologically relevant.

**Potential relevance:** These data also serve as a scientific basis for investigating the source of epizootics and outbreaks both nationally and internationally.
3.2 Introduction

Equine influenza (EI) is caused by a type A influenza virus of the *Orthomyxoviridae* family. The virus is highly contagious and EI is generally regarded as the most economically important respiratory disease of horses and other equidae. Equine influenza virus (EIV) spread in immunologically naïve populations is often explosive, resulting in substantial financial losses due to restriction of movement and cancellation of equestrian events. In 2007 the introduction of EIV into Australia for the first time resulted in the infection of more than 76,000 horses on over 10,000 premises in New South Wales and Queensland, costing a reported one billion Australian dollars (Garner et al., 2011). EI is endemic in Europe and North America where disease prevention and control rely heavily on the vaccination of highly mobile horses. Many countries, such as South Africa, India, Hong Kong, Dubai and Australia, where the disease is not endemic, have experienced epizootics of influenza associated with the importation of subclinically infected vaccinated horses (Guthrie et al., 1999; King and Macdonald 2004; Powell et al., 1995; Timoney 2000; Callinan 2008). The international movement of horses for sales, breeding or participation in competitions is an essential part of the Irish horse industry. Ireland is the third largest producer of Thoroughbreds in the world, accounting for over 42% of the EU output (Indecon Report, 2004). Bloodstock accounts for 10% of all livestock production in Ireland and in 2009 Irish horses were exported to 39 countries worldwide (HRI, 2009). Thus, it is essential that Ireland participates in the international EI monitoring and vaccine strain selection programme established by the World Organisation for Animal Health (OIE) in 1995.

Only two subtypes of EI (H7N7 and H3N8) have been associated with disease in the horse, but recently avian H5N1 was associated with respiratory disease in donkeys (Abdel-Moneim et al., 2010). The last confirmed outbreak caused by the H7N7 subtype was in 1979 (Webster, 1993). Viruses of the H3N8 subtype have been responsible for both widespread outbreaks in vaccinated horses and epizootics in immunologically naïve populations since they were first isolated in 1963. In the late 1980’s and early 1990’s the H3N8 subtype diverged into two distinct evolutionary lineages designated European and American (Daly et al., 1996). A later study of North American isolates indicated that the American lineage had further diverged into South American, Kentucky and Florida sublineages (Lai et al., 2001). Subsequent evolution within the
Florida sublineage has resulted in the emergence of two distinct clades namely clade 1 and clade 2. Clade 1 includes the A/eq/Wisconsin/1/03 and A/eq/South Africa/4/03-like viruses and clade 2 includes A/eq/Newmarket/5/03-like viruses. These variants are antigenically different from the original American lineage prototype A/eq/Newmarket/1/93. Clade 1 viruses have been isolated predominantly in the USA and associated with major outbreaks in South Africa 2003, Japan 2007 and Australia 2007 (Bryant et al., 2009; King and Macdonald 2004; Yamanaka et al., 2008; Callinan 2008). Clade 2 viruses have been isolated in Europe and associated with outbreaks in China in 2007 and 2008 and India in 2008 and 2009 (Bryant et al., 2009; Qi et al., 2010a; Virmani et al., 2010).

Antigenic drift of the H3N8 subtype impacts negatively on vaccine efficacy in that horses vaccinated with strains from one lineage require higher antibody levels to protect against strains from another lineage (Daly et al., 2004). It was proposed that four amino acid changes in at least two of the five putative antigenic sites on the HA protein result in antigenic drift (Wilson and Cox, 1990), which may render vaccines ineffective; however, more recent data suggests that a change of as few as two amino acids could result in significant antigenic drift (Jin et al., 2005). The last major EI epidemic in Ireland that occurred as a result of antigenic drift was in 1989. A mandatory vaccination programme for racehorses and competition horses had been in place for almost a decade at the time of the outbreak; however, analysis of the HA sequence of the causal virus indicated that it differed significantly from the strains contained in the vaccines (Nelly, 1996). In 2003 antigenic drift contributed to a large outbreak of EIV in Newmarket where over 1,300 vaccinated horses were affected (Newton et al., 2006). Recently, outbreaks of influenza were observed in Irish racing yards where the horses had been vaccinated with vaccines that had not been updated in accordance with the OIE recommendations of 2004; i.e. the vaccines did not contain a representative of the Florida sublineage (Gildea et al., 2011). In 2007 it was reported that several outbreaks of EI in the UK were associated with the importation of horses from Ireland (Bryant et al., 2009). Furthermore, the identification of clinically affected Irish stallions in an Australian quarantine station led to speculation that the virus responsible for the Australian outbreak might have originated in Ireland (Callinan, 2008). Antigenic and genetic drift of EI is monitored by the Expert Surveillance Panel (ESP), which includes scientists from the OIE and World Health Organisation (WHO).
reference laboratories. Data relating to outbreaks of influenza, particularly in vaccinated horses, and the antigenic and genetic characteristics of virus isolates are reviewed annually and recommendations are made on the need to update vaccines. Surveillance programmes are not only essential for this process to operate effectively but also serve to decrease the economic impact of EI by operating as an early warning system for the emergence and spread of genetic variants. This report provides a genetic analysis of viruses detected during EI outbreaks in Ireland from June 2007 until January 2010.

3.3 Materials and Methods

3.3.1 Sample collection and virus detection and isolation

Nasopharyngeal swabs were submitted to the Irish Equine Centre (IEC) by veterinary surgeons attending horses with clinical respiratory disease. After collection, the nasopharyngeal swabs were placed in 5ml of virus transport medium (VTM) consisting of phosphate buffered saline (PBS), 5000u/ml penicillin, 250mg/ml amphotericin B and 2% v/v foetal bovine serum. RNA was extracted from 140 µl of nasal secretions using the QIAamp Viral RNA Mini kit (Qiagen, Fleming Way, Crawley, West Sussex, UK) according to the manufacturer’s recommendations. One step RT-PCR was performed using Light Cycler RNA Amplification kit SYBR Green I (Roche Diagnostics, Charles Avenue, Burgees Hill, UK) as previously described (Quinlivan et al., 2005). Primers designed by Fouchier et al., (2000) were used to amplify a 244bp amplicon from nucleotide positions 32 to 276 of the matrix gene. RT-PCR positive samples (100 µl) were passaged up to four times in the allantoic cavities of 9 to 12 day-old embryonated hens’ eggs. Eggs were incubated at 34 ºC (+/- 1 ºC) for 48 hours and then placed at 4 ºC (+/- 1 ºC) overnight before harvesting. The allantoic fluid was tested for haemagglutination (HA) activity using 1% chicken erythrocytes in PBS (OIE, 2008). Positive samples were confirmed by haemagglutination inhibition (HI) with type-specific ferret antisera supplied by the National Institute of Biological Standards and Control (Potters bar, Hertfordshire, UK).
3.3.2 HA1 gene sequencing and analysis

Sequence data for the complete HA gene was generated for all viruses except A/eq/Meath/07 for which there was insufficient template. However, the majority of nucleotide substitutions that affect the antigenic structure of the HA occur in the HA1 gene which was the focus of this study. The sequence of the HA1 gene (1009bp) of 18 viruses identified in Ireland was determined. These included six viruses from 2007, one virus from 2008, eight viruses from 2009 and three viruses from 2010. The sequence data for 15 of these viruses were derived from viral RNA extracted from nasal swabs, the remaining three viruses (A/eq/Carlow/1/07, A/eq/Donegal/5/09, A/eq/Limerick/3/10) required amplification in embryonated hens’ eggs prior to sequencing. For these 18 viruses the HA gene was amplified by RT-PCR and sequenced in four separate reactions using the primers described in Table 3.1. The locations of the primer binding sites and product sizes are shown in Figure 3.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1F</td>
<td>AGCAAAAGCAGGGGATATTTTC</td>
</tr>
<tr>
<td>HA466R</td>
<td>GTGACACCTGTCCATGTG</td>
</tr>
<tr>
<td>HA359F</td>
<td>CAATTGCTACCCATATG</td>
</tr>
<tr>
<td>HA935R</td>
<td>GCTTCCATTGGGTTGTAAT</td>
</tr>
<tr>
<td>HA853F</td>
<td>TGAAAACAGGGAAAAGCTCTG</td>
</tr>
<tr>
<td>HA1195R</td>
<td>ATGGGCTGCTTGAAGTGCTT</td>
</tr>
<tr>
<td>HA1162F</td>
<td>AAGCTGCAATCTAAGA</td>
</tr>
<tr>
<td>HA1762R</td>
<td>AGTAGAAACAAGGGTGTTTTTAACTATC</td>
</tr>
<tr>
<td>HA203F</td>
<td>GAGCATTTCAAATGGGAAAA</td>
</tr>
<tr>
<td>HA359R</td>
<td>GGGATGTCATATGGGTAGCAA</td>
</tr>
<tr>
<td>HA492F</td>
<td>AGGGGATCAGCCGATAGTT</td>
</tr>
<tr>
<td>HA604R</td>
<td>GTGATGAATCCCCCAGATGT</td>
</tr>
</tbody>
</table>

Table 3.1: Primer sequences
Figure 3.1: Location of the primers listed in Table 3.1 with reference to a schematic diagram of the HA gene

Primers are numbered from the 5’ end.

For RT-PCR, RNA was reverse transcribed in a 20 µl reaction consisting of 1 µM of the appropriate F primer, 5 x AMV buffer (Roche Diagnostics), 1 unit of AMV RT (Roche Diagnostics), 200 µM of each dNTP (Applied Biosystems, Lingley House, 120 Birchwood Boulevard, Warrington, UK), 0.5µl RNAsin (Applied Biosystems) and 5 µl of viral RNA. Reverse transcription was carried out at 42°C for 60 min, followed by denaturation at 95°C for 5 min (G Storm Thermal cycler, Unit 3 Byfleet Technical Centre, Canada Road, Byfleet, Surrey, UK). A 50 µl PCR reaction contained 0.25 U AmpliTaq DNA polymerase (Applied Biosystems), 10 x Buffer (Applied Biosystems), 200 µM of each dNTP (Applied Biosystems), 0.5 µM of each primer and 10 µl cDNA. Denaturation was carried out at 95°C for 5 min, followed by amplification with 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 40 seconds with final elongation at 72°C for 5 min. RT-PCR products were analyzed on a 1.2 % agarose gel stained with ethidium bromide and purified using the QIAquick PCR Purification Kit (Qiagen). Sequencing was performed by Qiagen Sequencing Services (Germany) and analysis of the genetic sequence was undertaken with Lasergene software (DNAstar).
3.3.3 HA gene sequence analysis

Multiple nucleotide and amino acid sequence alignments were acquired using the CLUSTALW2 programme (Larkin et al., 2007) from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/). The HA1 gene sequences used in phylogenetic analysis, including the Irish viruses described here, are obtainable from the NCBI sequence database GenBank (see Table 3.2 for accession numbers). To determine the relationship between EI viruses, a phylogenetic tree was constructed. Phylogenetic analysis was constructed using with PHYLIP software v.3.68 (Felsenstein, 2005). Genetic matrix distances and branch lengths were calculated using DNADIST and FITCH respectively. Data were bootstrapped 100 times to assess the reliability of the phylogenetic tree (Hillis et al., 1993).

Table 3.2: EI viruses included in phylogenetic analysis

<table>
<thead>
<tr>
<th>Location</th>
<th>Lineage</th>
<th>Virus Name</th>
<th>Abbreviation</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Miami, USA</td>
<td>Prediv</td>
<td>A/eq/Miami/63</td>
<td>MIA/63</td>
<td>M29257</td>
</tr>
<tr>
<td>Fontainebleau, France</td>
<td>Prediv</td>
<td>A/eq/Fontainebleau/79</td>
<td>FON/79</td>
<td>CY032405</td>
</tr>
<tr>
<td>Newmarket, U.K.</td>
<td>Prediv</td>
<td>A/eq/Newmarket/79</td>
<td>NM/79</td>
<td>D30677</td>
</tr>
<tr>
<td>Kentucky, USA</td>
<td>Prediv</td>
<td>A/eq/Kentucky/2/81</td>
<td>KY/2/81</td>
<td>CY028820</td>
</tr>
<tr>
<td>Kildare, Ireland</td>
<td>Eu</td>
<td>A/eq/Kildare/89</td>
<td>KIL/89</td>
<td>JN222941</td>
</tr>
<tr>
<td>Hong Kong</td>
<td>Eu</td>
<td>A/eq/Hong-Kong/1/92</td>
<td>HK/92</td>
<td>L27597</td>
</tr>
<tr>
<td>Kentucky, USA</td>
<td>Am</td>
<td>A/eq/Kentucky/1/92</td>
<td>KY/1/92</td>
<td>CY030149</td>
</tr>
<tr>
<td>Kildare, Ireland</td>
<td>Am</td>
<td>A/eq/Kildare/92</td>
<td>KIL/92</td>
<td>JN084402</td>
</tr>
<tr>
<td>Newmarket, U.K.</td>
<td>Am</td>
<td>A/eq/Newmarket/1/93</td>
<td>NM/1/93</td>
<td>X85088</td>
</tr>
<tr>
<td>Newmarket, U.K.</td>
<td>Eu</td>
<td>A/eq/Newmarket/2/93</td>
<td>NM/2/93</td>
<td>X85089</td>
</tr>
<tr>
<td>Kentucky, USA</td>
<td>Am</td>
<td>A/eq/Kentucky/1/97</td>
<td>KY/1/97</td>
<td>AF197249</td>
</tr>
<tr>
<td>Kentucky, USA</td>
<td>Am</td>
<td>A/eq/Kentucky/1/98</td>
<td>KY/1/98</td>
<td>AF197241</td>
</tr>
<tr>
<td>Ohio, USA</td>
<td>FC1</td>
<td>A/eq/Ohio/1/03</td>
<td>OHI/1/03</td>
<td>DQ124192</td>
</tr>
<tr>
<td>Wisconsin, USA</td>
<td>FC1</td>
<td>A/eq/Wisconsin/1/03</td>
<td>WIS/1/03</td>
<td>DQ222913</td>
</tr>
<tr>
<td>Location</td>
<td>Code</td>
<td>Accession Information</td>
<td>Date</td>
<td>GenBank Accession</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------</td>
<td>-----------------------</td>
<td>-------</td>
<td>------------------</td>
</tr>
<tr>
<td>Newmarket, U.K.</td>
<td>FC2</td>
<td>A/eq/Newmarket/5/03</td>
<td>NM/5/03</td>
<td>FJ375213</td>
</tr>
<tr>
<td>Kentucky, USA</td>
<td>FC1</td>
<td>A/eq/Kentucky/9/04</td>
<td>KY/9/04</td>
<td>FJ195451</td>
</tr>
<tr>
<td>Aboyne, Scotland</td>
<td>Eu</td>
<td>A/eq/Aboyne/1/05</td>
<td>ABY/05</td>
<td>EF541442</td>
</tr>
<tr>
<td>Kildare, Ireland</td>
<td>FC2</td>
<td>A/eq/Kildare/07</td>
<td>KIL/07</td>
<td>JN222936</td>
</tr>
<tr>
<td>Donegal, Ireland</td>
<td>FC2</td>
<td>A/eq/Donegal/07</td>
<td>DNG/07</td>
<td>JN222934</td>
</tr>
<tr>
<td>Meath, Ireland</td>
<td>FC2</td>
<td>A/eq/Meath/1/07</td>
<td>MTH/1/07</td>
<td>JN222935</td>
</tr>
<tr>
<td>Richmond, U.K.</td>
<td>FC2</td>
<td>A/eq/Richmond/1/07</td>
<td>RIC/1/07</td>
<td>FJ195395</td>
</tr>
<tr>
<td>Ibaraki, Japan</td>
<td>FC1</td>
<td>A/eq/Ibaraki/1/07</td>
<td>IBA/1/07</td>
<td>AB360549</td>
</tr>
<tr>
<td>Pennsylvania, USA</td>
<td>FC1</td>
<td>A/eq/Pennsylvania/1/07</td>
<td>PEN/1/07</td>
<td>FJ195406</td>
</tr>
<tr>
<td>Lincolnshire, U.K.</td>
<td>FC1</td>
<td>A/eq/Lincolnshire/1/07</td>
<td>LIN/1/07</td>
<td>FJ195398</td>
</tr>
<tr>
<td>Down, Ireland</td>
<td>FC2</td>
<td>A/eq/Down/08</td>
<td>DWN/1/08</td>
<td>JN222937</td>
</tr>
<tr>
<td>Donegal, Ireland</td>
<td>FC1</td>
<td>A/eq/Donegal/1/09</td>
<td>DNG/09</td>
<td>JN222938</td>
</tr>
<tr>
<td>Carlow, Ireland</td>
<td>FC1</td>
<td>A/eq/Carlow/09</td>
<td>CLW/09</td>
<td>JN222939</td>
</tr>
<tr>
<td>Limerick, Ireland</td>
<td>FC1</td>
<td>A/eq/Limerick/1/10</td>
<td>LIM/10</td>
<td>JN222940</td>
</tr>
</tbody>
</table>


### 3.4 Results

#### 3.4.1 Genetic characterisation and phylogeny

The accession numbers of EI viruses identified in Ireland during the study period are listed in Table 3.2. There were no HA1 nucleotide sequence differences between RNA extracted directly from swab material and RNA extracted from allantoic fluid of infected eggs from samples taken on the same premises (data not shown). Phylogenetic analysis using the sequences obtained in this study and reference strains from Genbank grouped all viruses into five well-supported clades comprising the Pre-divergent, Eurasian and American lineages including the Florida sublineage clades 1 and 2 (Figure 3.2). The vaccination status of the horses and the type of premises where the viruses were detected during this study are summarised in Table 3.3. The HA1 nucleotide sequence of four (A/eq/Meath/07, A/eq/Laois/07, A/eq/Carlow/1/07,
A/eq/Carlow/2/07) of the six viruses isolated on separate premises in 2007 were identical. A/eq/Donegal/1/09 was representative of A/eq/Donegal/2/09, A/eq/Donegal/3/09, A/eq/Donegal/4/09, A/eq/Donegal/5/09, A/eq/Donegal/6/09 and A/eq/Wexford/09 identified on four different premises and A/eq/Limerick/1/10 was representative of A/eq/Limerick/2/10 and A/eq/Limerick/3/10 which were identified on a single premises. Analysis of EI viruses identified in Ireland from 2007-2008 indicated that they were members of the clade 2 Florida sublineage (Figure 3.2). Analysis of EI viruses identified in Ireland from November 2009 to January 2010 indicated that they were grouped within the clade 1 Florida sublineage. This was the first time viruses of the clade 1 Florida sublineage were identified in Ireland.
Figure 3.2: Phylogenetic tree of HA1 nucleotide sequences (n=18)

Phylogenetic analysis of the HA1 nucleotide sequences encoded by EIV, subtype H3N8. Bootstrap values obtained after 100 replicates are shown at the major nodes. Phylogenetic groups are shown by continuous bars on the right and are labelled as appropriate. Accession numbers for the genes reported in this manuscript are listed in Table 3.2. Black = pre-divergent; blue = Eurasian; red = American; green = Florida sublineage clade 1; purple = Florida sublineage clade 2. From the starting signal sequence A/eq/Meath/1/07 is representative of A/eq/Laois/07, A/eq/Carlow/1/07 and A/eq/Carlow/2/07. A/eq/Donegal/1/09 is representative of A/eq/Donegal/2/09, A/eq/Donegal/3/09, A/eq/Donegal/4/09, A/eq/Donegal/5/09, A/eq/Donegal/6/09 and A/eq/Wexford/09. A/eq/Limerick/1/10 is representative of A/eq/Limerick/2/10 and A/eq/Limerick/3/10.
Table 3.3: EI viruses detected in Ireland 2007-2010: Type of premises and reported vaccination status of horses

<table>
<thead>
<tr>
<th>Virus</th>
<th>Clade</th>
<th>Virus isolated</th>
<th>Date</th>
<th>Type of premise</th>
<th>Vaccination Status†</th>
<th>Vaccination status of EI positive horse</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/eq/Laois/07</td>
<td>2</td>
<td>Yes</td>
<td>June ’07</td>
<td>Polo pony yard</td>
<td>0%</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/07</td>
<td>2</td>
<td>Yes</td>
<td>July ’07</td>
<td>Non TB farm</td>
<td>0%</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Meath/1/07</td>
<td>2</td>
<td>Yes</td>
<td>July ’07</td>
<td>Racing yard</td>
<td>89.3%</td>
<td>Out of date</td>
</tr>
<tr>
<td>A/eq/Carlow/1/07</td>
<td>2</td>
<td>Yes</td>
<td>Aug. ’07</td>
<td>Racing yard</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>A/eq/Carlow/2/07</td>
<td>2</td>
<td>Yes</td>
<td>Aug. ’07</td>
<td>Racing yard</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>A/eq/Kildare/07</td>
<td>2</td>
<td>Yes</td>
<td>Aug. ’07</td>
<td>Show jumping yard</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>A/eq/Down/08</td>
<td>2</td>
<td>Yes</td>
<td>May ’08</td>
<td>TB public stud</td>
<td>Described by V.S. as mixed</td>
<td>Unknown</td>
</tr>
<tr>
<td>A/eq/Wexford/09</td>
<td>1</td>
<td>No</td>
<td>Nov. ’09</td>
<td>Hunting yard</td>
<td>0%</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/1/09</td>
<td>1</td>
<td>No</td>
<td>Dec. ’09</td>
<td>Riding School*</td>
<td>0% (3/20 horses described by V.S. as vaccinated)</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/2/09</td>
<td>1</td>
<td>Yes</td>
<td>Dec. ’09</td>
<td>Riding School*</td>
<td>0% (3/20 horses described by V.S. as vaccinated)</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/3/09</td>
<td>1</td>
<td>Yes</td>
<td>Dec. ’09</td>
<td>Riding School*</td>
<td>0% (3/20 horses described by V.S. as vaccinated)</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/4/09</td>
<td>1</td>
<td>Yes</td>
<td>Dec. ’09</td>
<td>Non TB farm</td>
<td>0%</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/5/09</td>
<td>1</td>
<td>Yes</td>
<td>Dec. ’09</td>
<td>Riding School*</td>
<td>0% (3/20 horses described by V.S. as vaccinated)</td>
<td>Unvaccinated</td>
</tr>
<tr>
<td>A/eq/Donegal/6/09</td>
<td>1</td>
<td>No</td>
<td>Dec. ’09</td>
<td>Show</td>
<td>0%</td>
<td>Unvaccinated</td>
</tr>
</tbody>
</table>
### 3.4.2 Amino acid alignment

An alignment of the HA1 gene of EI viruses identified in Ireland and representative viruses (clade 1 and 2) was carried out and any amino acid changes are summarised in Figure 3.3. The numbering of the HA1 sequence starts with the serine residue immediately downstream of the predicted signal peptide cleavage site (Hae et al., 2003). Negative numbers represent the predicted signal sequence. All Irish viruses identified in 2007-2008 belonged to clade 2 of the Florida sublineage and did not have the two characteristic amino acid substitutions (V78A and N159S) in putative antigenic sites that distinguish viruses like A/eq/Wisconsin/1/03 and A/eq/South Africa/4/03 (clade 1 viruses) from the UK prototype clade 2 A/eq/Newmarket/5/03 virus (Newton et al., 2006). They had the “FIFI” motif at position -11 to -8 first identified in UK isolates in 2004 (Bryant et al., 2009). In addition, they all had an amino acid substitution G7N when compared to A/eq/Newmarket/5/03. Within the 2007 Irish isolates a further three amino acid substitutions occurred and are illustrated in Figure 3.3. One of these, Y161H, which occurred in A/eq/Donegal/07, was in antigenic site B (Qi et al., 2010b). The two remaining changes were S91N in A/eq/Kildare/07 and D291E in A/eq/Kildare/07 and A/eq/Meath/07. The virus A/eq/Down/08 had two amino acid changes N9S and E291D in comparison to the 2007 Irish representative virus A/eq/Meath/07. Neither of these occurred in antigenic sites; however, the latter amino acid substitution was previously detected in the 2007 isolate A/eq/Donegal/07. HA2 amino acid sequence analysis of six of the seven clade 2 viruses included in this study.

![Table]

<table>
<thead>
<tr>
<th>Virus</th>
<th>Vaccination</th>
<th>Date</th>
<th>Location</th>
<th>Vaccination Status</th>
<th>HA1 Status</th>
<th>Vaccination Rate</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/eq/Carlow/09</td>
<td>1</td>
<td>No</td>
<td>Dec. '09</td>
<td>Non TB farm</td>
<td>0%</td>
<td>Unvaccinated</td>
<td></td>
</tr>
<tr>
<td>A/eq/Limerick/1/10</td>
<td>1</td>
<td>No</td>
<td>Jan. '10</td>
<td>Racing yard</td>
<td>43.5%</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>A/eq/Limerick/2/10</td>
<td>1</td>
<td>No</td>
<td>Jan. '10</td>
<td>Racing yard</td>
<td>43.5%</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>A/eq/Limerick/3/10</td>
<td>1</td>
<td>Yes</td>
<td>Jan. '10</td>
<td>Racing yard</td>
<td>43.5%</td>
<td>Unknown</td>
<td></td>
</tr>
</tbody>
</table>

† = % up to date vaccination records available; TB = Thoroughbred; Aug. = August; Nov. = November; Dec. = December; Jan. = January; V.S. = Veterinary Surgeon; * = same riding school; ♦ = same racing yard. Some of these data were included in a previous publication relating to the management and environmental factors involved in equine influenza outbreaks in Ireland (Gildea et al., 2011).
indicated that they were identical except for one amino acid change G464D in A/eq/Donegal/07.

Ten of the eleven viruses belonging to the Florida sublineage clade 1 identified on five premises in Ireland (riding school, non-Thoroughbred farm, show jumping yard, hunting yard and racing yard) from 2009 to 2010 were identical from the serine residue immediately downstream of the predicted signal peptide cleavage site. Sequence analysis of the region upstream of the signal sequence indicated that three viruses identified on a premises in County Donegal in 2009, one virus identified on a premises in County Carlow in 2009 and two viruses identified on a premises in County Limerick in 2010 were identical. Two of these viruses (A/eq/Donegal/5/09, A/eq/Limerick/3/10) required amplification in embryonated hens’ eggs prior to sequencing. In this region upstream of the signal sequence, they had two amino acid substitutions in comparison to A/eq/Wisconsin/1/03 (K-16T, T-15A). A further six amino acid substitutions in comparison to A/eq/Wisconsin/1/03 were identified. Two were in antigenic sites A and E (A138S, R62K) (Lai et al., 2001; Qi et al., 2010b). The remaining four amino acid substitutions are illustrated in Figure 3.3 and occurred at the following positions G7D, S45G, D104N and V223I. A/eq/Carlow/09 had an additional amino acid substitution N3K when compared to all other Irish viruses. HA2 amino acid sequence analysis of clade 1 viruses included in this study indicated that they were identical except for two amino acid changes, one of which occurred in A/eq/Wexford/09 (F447L) and one of which occurred in A/eq/Limerick/3/10 (G510S).
Chapter 3 Experimental

Figure 3.3: HA1 amino acid sequence alignment

3.5 Discussion

The genetic analysis of Irish EI viruses identified from June 2007 to January 2010 was carried out as part of the international EI monitoring programme. The results of this surveillance are presented annually to the ESP. The criteria for updating EI vaccines are similar to those for human influenza vaccines and based on analysis of evidence of disease in well-vaccinated horses, antigenic changes, genetic changes and, when available, experimental challenge data. The genetic analysis is currently based on the sequencing of the HA1 gene. The sequence data from this study demonstrate that viruses from the Florida sublineage clades 1 and 2 circulated in Ireland from 2007 to 2010. The EI viruses in approximately 16% of RT-PCR positive nasal swabs collected during the study period were characterised. All of these viruses were detected in the nasal secretions of unvaccinated horses, horses of unknown vaccination history or, in one instance, a horse that had not been vaccinated for 15 months prior to sampling. This suggests that unvaccinated horses are the best source of virus for strain characterisation. Equine influenza virus can be detected in infected seropositive vaccinated horses by RT-PCR but such animals tend to shed less virus and thus provide insufficient PCR template for sequence analysis.

Bryant et al. (2009; 2011) previously described a predominance of Florida sublineage clade 2 viruses in circulation in Europe from 2006 to 2008 and this was consistent with our findings for 2007 and 2008. Amino acid alignments of the viruses identified in this study with A/eq/Wisconsin/1/03 and A/eq/Newmarket/5/03, representatives of clade 1 and clade 2, respectively, established that changes V78A and N159S were consistent between the two clades (Figure 3.3). Five amino acid changes between the clade 2 viruses isolated in this study and A/eq/Newmarket/5/03 were noted. One of these substitutions (Y161H) in A/eq/Donegal/07 was located in antigenic site B (Qi et al., 2010b). To our knowledge this amino acid substitution has not previously been reported in any equine H3N8 virus to date. Antigenic site B is positioned at the most external fragment of the HA1 loop and amino acid changes in this site are highly likely to influence viral antigenicity (Daniels et al., 1983; Jin et al., 2005). Of the remaining four amino acid substitutions, G7N was found in Florida clade 2 viruses recently identified in the UK, China, Mongolia and India and D291E was observed in viruses identified in the UK in 2006 and 2007 but not in viruses identified in China, Mongolia
In 2007 several outbreaks of EI were reported in the UK where the index cases were horses recently imported from Ireland. There is constant movement of horses between Ireland and the UK for breeding, competition, sales and other purposes. As EI is endemic, it is not unusual for the same EI viruses to circulate in both countries (Nelly, 1996). Furthermore, it has been suggested that the stress associated with travel may make horses more susceptible to infection (Bryant et al., 2009). The representative virus A/eq/Meath/1/07 which was identified on four of the six premises where EI outbreaks occurred in 2007 displayed 100% nucleotide identity to a UK isolate A/eq/Richmond/1/07. In a study carried out on viruses isolated in the UK from 2006 to 2007, A/eq/Richmond/1/07 was described as being representative of 10 viruses (Bryant et al., 2009). It was reported that five of these viruses were isolated in four foci of EIV infection where the index cases were animals that had been imported into the UK having being bought at a horse sale in County Kilkenny (Newton et al., 2007). It was proposed that A/eq/Richmond/1/07 was therefore likely to be at least partially representative of the viruses circulating in Ireland at that time (Bryant et al., 2009). This suggests conjecture in the UK that the horse sale in Ireland was the source of the virus. However, it should be noted that this sale took place on the 26th of May 2007 and that A/eq/Lanark/1/06 and A/eq/Horsham/1/07 were isolated in the UK many months previously (Bryant et al., 2009). Thus, this virus was circulating in the UK prior to the arrival of the horses purchased at the horse sale in County Kilkenny. Following notification of outbreaks of EI in the UK that involved recently imported horses from Ireland, a notice was sent by the IEC to over 400 veterinary clinicians appraising them of the situation, requesting samples from suspect cases and offering to supply sampling kits and laboratory testing free of charge. No outbreaks of respiratory disease linked to the movement of horses from the horse sale in Kilkenny were identified in Ireland. The first A/eq/Richmond/1/07 like virus identified in Ireland was detected in polo ponies in June 2007.
Previous experience has highlighted that the index case during an outbreak may not necessarily be the original source of virus. In Australia 2007, the first recorded clinical case at the Eastern Creek quarantine station where the EI outbreak began was an Irish horse (Callinan, 2008). This led to speculation that the virus had originated in Ireland. Subsequently, virological and epidemiological evidence indicated that the source of the clade 1 virus responsible for the outbreak was a horse imported from Japan. It is probable that the virus had originated in the United States as the Japanese and Australian virus isolates (A/eq/Ibaraki/07 and A/eq/Sydney2888-8/07) are genetically related to A/eq/Pennsylvania/1/07. Such clade 1 viruses have predominated in North America for several years but became more widespread in the UK in 2009 (Bryant et al., 2011). In 2009, the first clade 1 virus was identified in Ireland and several outbreaks of EI from November 2009 to January 2010 were attributed to clade 1 viruses.

In Ireland seven of the eight clade 1 viruses identified on four premises in 2009 and all three viruses identified on a single premises in 2010 were identical. Horses from two of the affected premises in 2009 had attended the same training show. In total, eight amino acid changes occurred between all clade 1 viruses identified in Ireland and the prototype clade 1 virus A/eq/Wisconsin/1/03. Two amino acid substitutions occurred in the predicted signal sequence at K-16T and T-15A. The single substitution K-16T was initially identified in a Canadian isolate A/eq/Guelph/03 and has since been identified in clade 1 viruses from the USA and the UK (Bryant et al., 2009), Japan and Egypt (GenBank). However, this appears to be the first report highlighting the two amino acid substitutions K-16T and T-15A in the predicted signal sequence of EI H3N8 viruses. Of the six remaining amino acid substitutions, five (G7D, R62K, D104N, A138S and V223I) are present in recent isolates from the UK, Egypt and the USA but not in the virus responsible for the epizootic in Japan in 2007 (A/eq/Ibaraki/07) (Bryant et al., 2011). The substitution S45G was first reported in viruses isolated in the UK in 2009 (Bryant et al., 2011) indicating the close similarity between the Irish and UK isolates at this time. An additional amino acid substitution N3K was identified in a virus A/eq/Carlow/09 detected on a single premises.
In conclusion, EI surveillance has demonstrated that from June 2007 to January 2010 viruses of the Florida sublineage were circulating in Ireland. While clade 2 viruses predominated up to November 2009, thereafter, clade 1 viruses were responsible for all the outbreaks identified. No virus of the Eurasian lineage was isolated during this surveillance period. In 2010 the ESP recommended that the vaccines should not include an H7N7 virus or an H3N8 virus of the Eurasian lineage but that they should contain both a clade 1 and clade 2 virus of the Florida sublineage (OIE, 2010). Antigenic characterisation has demonstrated that clade 2 viruses are clearly different from clade 1 viruses and there is some evidence that vaccines containing earlier viruses of the American lineage such as A/eq/Newmarket/1/93 do not provide adequate protection against these viruses (Bryant et al., 2009; 2011; Gildea et al., 2011). The surveillance data presented here support the ESP recommendations and indicate that they are epidemiologically relevant.

Acknowledgements
The experimental work was carried out at the Irish Equine Centre and funded by the Department of Agriculture under the National Development Plan. The results will be submitted as part of a PhD thesis by Sarah Gildea to the University of Limerick.

3.6 References


King, E. L., Macdonald, D (2004) 'Report of the Board of Inquiry appointed by the Board of the National Horseracing Authority to conduct enquiry into the causes of the equine influenza which started in the Western cape in early December


Chapter 4
A Comparative Antibody Study of the Potential Susceptibility of Thoroughbred and Non-Thoroughbred Horse Populations in Ireland to Equine Influenza Virus


Authors: Sarah Gildea, Sean Arkins, Ann Cullinane
4.1 Abstract

**Background:** In Ireland horses may be protected against equine influenza virus (EIV) as a result of natural exposure or vaccination. Current mandatory vaccination programmes are targeted at horses that engage in competition or breeding activities and thus travel nationally and/or internationally. A correlation between antibody levels as measured by single radial haemolysis (SRH) and protective immunity against EIV has been established.

**Objectives:** The objective of this study was to determine the susceptibility of selected populations of horses by quantifying their antibodies to EIV.

**Methods:** Blood samples were collected from Thoroughbred weanlings, yearlings, racehorses and broodmares, teaser stallions and non-Thoroughbred horses. Antibodies against EIV H3N8 and H7N7 were measured by SRH.

**Results:** The order of susceptibility to equine influenza (EI) in the populations examined in Ireland was as follows: Thoroughbred weanlings > Teasers > non-Thoroughbred horses and ponies > Thoroughbred yearlings > Thoroughbred horses in training > Thoroughbred broodmares.

The H3N8 antibody levels of the weanlings, yearlings, broodmares and horses in training were similar to their H7N7 antibody levels suggesting that their antibodies were primarily vaccinal in origin. The teasers and non-Thoroughbreds had higher H3N8 antibody levels than H7N7 antibody levels suggesting that the majority of seropositive horses in these populations had been exposed to H3N8 by natural infection.

**Conclusions:** Weanlings, teasers and non-Thoroughbred horses were identified as most susceptible to EIV. The results suggest that it would be advisable that weanlings are vaccinated prior to attendance at public sales, that teaser stallions are vaccinated prior to each breeding season and that mandatory vaccination should be implemented for participation in non-Thoroughbred events.
4.2 Introduction

Equine influenza virus (EIV) of the H3N8 subtype has long been regarded as the most important respiratory pathogen of horses due to its highly contagious nature and rapid spread among susceptible animals (Timoney, 1996). The introduction of EIV to immunologically naïve populations has resulted in substantial financial losses due to restriction of movement and the cancellation of race meetings and other equestrian events. In South Africa in 1986 (Guthrie et al., 1999) and in Hong Kong in 1992 (Powell et al., 1992) racing was suspended for five months and one month respectively following an incursion of EIV. The introduction of EIV into Australia for the first time in 2007 resulted in the infection of over 76,000 horses on over 10,000 premises in New South Wales and Queensland, costing a reported one billion Australian dollars (Garner et al., 2011). At present the only countries that have never experienced an incursion of EIV are New Zealand and Iceland. The virus is endemic in Europe and America where outbreaks of disease result in financial loss due to disruption of training schedules.

Large outbreaks of equine influenza (EI) are often associated with the congregation of horses at equestrian events and their subsequent dispersal over a wide geographical area (van Maanen and Cullinane, 2002). In endemic countries the economic losses due to EI can be minimised by targeted vaccination of highly mobile horses. In Ireland the Turf Club implements a mandatory vaccination programme for racehorses and vaccination is also required for yearlings and horses in training, prior to entry to the major Thoroughbred sales. All horses participating in Federation Equestre Internationale (FEI) competitions must be vaccinated and several horse and pony societies have a mandatory vaccination policy. The overall aim of such vaccination policies is to ensure sufficient herd immunity to protect equestrian events rather than individual horses. Mandatory vaccination of racehorses and competition horses was introduced in Ireland in 1981. Despite sporadic outbreaks and one countrywide epidemic in 1989, this policy has been successful as no major equestrian event has been cancelled due to EI for almost three decades (Cullinane et al., 2009). This is similar to the situation in the United Kingdom (Newton et al., 2006).

In Ireland horses may be protected against EI as a result of exposure to virus by natural infection or by vaccination. A definitive correlation between antibody levels against the
virus haemagglutinin protein as measured by the Single Radial Haemolysis (SRH) test, and protective immunity against EI has been established in both experimental challenge studies and the field (Mumford and Wood, 1992; Mumford et al., 1994; Townsend et al., 1999; Newton et al., 2000a; Mumford, 2001). Published data suggest that horses with antibody levels of 85mm$^2$ or greater are clinically protected against EIV and that those with antibody levels of 150mm$^2$ or greater are virologically protected and do not shed virus after challenge (Mumford et al., 1994; Newton et al., 2000a; Mumford, 2001). Horses with antibody levels of less than 50mm$^2$ are 15 times more likely to be the index case within a yard and represent a significant risk to other horses (Wood, 1991).

There are five EI vaccines currently commercially available in Ireland; two inactivated whole virus vaccines, two subunit vaccines and a canarypox recombinant vaccine. The kinetics of the antibody response to these vaccines is similar (Gildea et al., 2011a). The cold-adapted modified live EI vaccine available in the United States, which does not induce antibodies correlating with protection (Townsend et al., 2001; Lunn et al., 2001), is not available in Ireland. All five available vaccines contain H3N8 viruses from the American and Eurasian lineages. The inactivated and subunit vaccines that are administered to the majority of vaccinated horses in Ireland contain a representative H7N7 virus that is not included in the canarypox vaccine (Toulemonde et al., 2005). Although H7N7 viruses co-circulated with H3N8 viruses in horses for many years, these viruses have not been isolated for over two decades and are considered to be extinct (Webster et al., 1993). The World Organisation for Animal Health or OIE (Office International des Epizooties) stipulates that there is no requirement for inclusion of an H7N7 virus in equine influenza vaccines (OIE, 2008a). However, antibodies against this virus are a useful aid in the differentiation of vaccinated horses from naturally infected horses.

The objective of this study was to determine the susceptibility of selected populations of horses in Ireland to EI by quantifying their antibodies to EIV H3N8. It was not possible to differentiate between H3N8 antibodies due to natural exposure and vaccination but the measurement of antibodies against H7N7 provided information on the populations vaccinated with any of the four commercially available vaccines containing a representative of that subtype.
4.3 Materials and Methods

4.3.1 Samples collection and horse populations

Whole blood samples (5 to 10ml) were collected for analysis by SRH from the populations listed below. The vaccination histories of the horses included in this study were unknown. No diagnosis of EIV was made on any of the selected premises where samples were collected and there was no overlap between premises or animals in any of the groups listed.

**Thoroughbred Weanlings:** Two hundred blood samples were collected. There were collected from five weanlings on each of 40 premises located in 11 of the 32 counties in Ireland.

**Thoroughbred Yearlings:** Two hundred and fifteen blood samples were collected. There were collected from five yearlings on each of 43 premises in 10 counties.

**Thoroughbred Broodmares:** Two hundred and five blood samples were collected. There were collected from five broodmares on each of 41 premises in 10 counties. (Analysis of 131 broodmares in this population revealed a minimum and maximum age of 4 and 25 years respectively and a mean age of 9.44 +/- 0.364 SE years).

**Teaser Stallions:** Blood samples were collected from 70 teaser stallions on 50 premises in 10 counties. These horses were of varying age but all sexually mature (greater than 2 years of age) as teaser stallions are used to determine if mares are in oestrus.

**Thoroughbred Racehorses:** Blood samples were collected from 233 Flat and National Hunt (Jump) horses on 53 premises in 16 counties. (Analysis of 182 horses in this population revealed a minimum and maximum age of 2 and 9 years respectively and a mean age 3.61 +/- 0.111 SE years).

**Non-Thoroughbred:** Two hundred and thirty six samples were collected from non-Thoroughbred horses and ponies of varying age, gender and discipline on 120 premises in 10 different counties in Ireland.
4.3.2 Serology
Antibodies against A/eq/Newmarket/2/93 (H3N8) a representative of the Eurasian lineage, A/eq/Kildare/92 (H3N8) a representative of the American lineage, A/eq/South Africa/4/03 (H3N8) a representative of the Florida sub-lineage of the American lineage and A/eq/Prague/56, the prototype H7N7 virus, were measured using the SRH test according to standard procedures (OIE, 2008b). In brief, optimized viral antigens were coupled to sheep red blood cells (SRBC) with chromium chloride. Agarose plates (1% wt/vol) were prepared containing the sensitized SRBC’s and guinea pig complement. The test sera were heat inactivated at 56 °C ± 1 °C for 30 minutes, aliquoted in 10 µl volumes into wells cut in the agarose plate and the plates were then incubated at 34°C ± 1°C for 20 to 24 hours.

Control antisera against A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/2/93 (H3N8), A/eq/Newmarket/1/93 (H3N8) and A/eq/South Africa/4/03 (H3N8) from the European Directorate for the Quality of Medicines and Healthcare (EDQM), were included on each plate as appropriate. The haemolytic zones resulting from the lysis of the antigen-coated SRBC’s by the antibody in the test sera were measured with a viewer and digital recording apparatus (Mitutoya, Aurora, Illinois, USA). The area of haemolysis was calculated and results were expressed in mm². The results were interpreted in accordance with published data; horses with antibody levels of 150mm² or greater were classified as virologically protected, horses with antibody levels less than 150mm² but greater than 85mm² were classified as clinically protected, horses with antibody levels less than 85mm² but greater than 50mm² were classified as partially protected and horses with antibody levels less than 50mm² were classified as potential index cases (Wood et al., 1991; Mumford, 2001).

4.3.3 Statistical analysis
SPSS version 16.0 for Windows (Chicago, Illinois, USA) was used to analyse the data. Mean H3N8 antibody values were calculated from SRH results obtained against the subtype 2 antigens A/eq/Kildare/92, A/eq/Newmarket/2/93 and A/eq/South Africa/4/03. ANOVA, Post Hoc Boniferroni and independent T tests were carried out where appropriate to compare mean antibody levels in the equine populations included in this study. The correlation between H7N7 and H3N8 antibodies in horses that were
seropositive to H7N7 was examined using Pearson’s correlation test. The H3N8 antibody levels of horses that were seropositive for both H3N8 and H7N7 were compared with those of horses that were seronegative for H7N7 by a one sample T test (Petrie and Watson, 1999). A significance level of p< 0.05 was used for all statistical tests.

### 4.4 Results

Descriptive statistics of equine influenza H3N8 and H7N7 antibody results obtained from the six populations examined are displayed in Table 4.1 and significant differences between the groups are illustrated in Figures 4.1 and 4.2. The weanlings had similar mean H3N8 and H7N7 antibody levels. This was also true for the yearlings, broodmares and horses in training. The teasers and non-Thoroughbreds had significantly higher H3N8 antibody levels than H7N7 antibody levels (p <0.01).

There was a significant difference in mean H3N8 antibody levels between all of the populations except in the case of yearlings, non-Thoroughbreds and teasers. There was a significant difference in mean H7N7 antibody levels between all of the populations except in the case of weanlings, non-Thoroughbreds and teasers. Tables 4.2 and 4.3 summarise the number of horses in each population with different levels of antibody (<50 mm$^2$, 50-85 mm$^2$, 85-150 mm$^2$ and >150 mm$^2$) and Tables 4.4a and 4.4b summarise the distribution (number of premises) of weanlings, yearlings and broodmares with different levels of antibodies.

The H3N8 antibody levels of horses that were seropositive to both subtypes correlated significantly with their H7N7 antibody levels (p <0.01). The H3N8 antibody levels of horses that were seropositive for both H3N8 and H7N7 were significantly higher than the H3N8 antibody levels of horses that were seronegative for H7N7 (p <0.01).
Table 4.1: Descriptive Statistics of H3N8 and H7N7 antibody results

<table>
<thead>
<tr>
<th></th>
<th>H3N8 SRH (mm²)</th>
<th>H7N7 SRH (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weanling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=200</td>
<td>Mean 14.3 ± 2.61 SE</td>
<td>Mean 12.8 ± 2.77SE</td>
</tr>
<tr>
<td></td>
<td>Median 0.0</td>
<td>Median 0.0</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 36.88</td>
<td>Std. Deviation 39.21</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 218.4</td>
<td>Maximum 182.2</td>
</tr>
<tr>
<td><strong>Yearling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=215</td>
<td>Mean 94.4 ± 5.34 SE</td>
<td>Mean 97.5 ± 6.19 SE</td>
</tr>
<tr>
<td></td>
<td>Median 91.8</td>
<td>Median 102.5</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 78.31</td>
<td>Std. Deviation 90.80</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 278.7</td>
<td>Maximum 305.2</td>
</tr>
<tr>
<td><strong>Broodmare</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=205</td>
<td>Mean 143.2 ± 3.64 SE</td>
<td>Mean 176.3 ± 4.27 SE</td>
</tr>
<tr>
<td></td>
<td>Median 146.1</td>
<td>Median 178.5</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 52.13</td>
<td>Std. Deviation 61.14</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 276.6</td>
<td>Maximum 285.3</td>
</tr>
<tr>
<td><strong>Teaser</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=70</td>
<td>Mean 65.7 ± 6.80 SE</td>
<td>Mean 30.9 ± 7.80 SE</td>
</tr>
<tr>
<td></td>
<td>Median 63.7</td>
<td>Median 65.24</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 56.90</td>
<td>Std. Deviation 65.24</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 240.2</td>
<td>Maximum 222.1</td>
</tr>
<tr>
<td><strong>Horses in Training</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=233</td>
<td>Mean 121.8 ± 3.30 SE</td>
<td>Mean 143.3 ± 4.54 SE</td>
</tr>
<tr>
<td></td>
<td>Median 123.5</td>
<td>Median 154.9</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 49.35</td>
<td>Std. Deviation 67.82</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 283.3</td>
<td>Maximum 268.6</td>
</tr>
<tr>
<td><strong>Non-Thoroughbred</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=236</td>
<td>Mean 84.3 ± 4.38 SE</td>
<td>Mean 30.2 ± 4.13 SE</td>
</tr>
<tr>
<td></td>
<td>Median 87.7</td>
<td>Median 63.40</td>
</tr>
<tr>
<td></td>
<td>Std. Deviation 67.33</td>
<td>Std. Deviation 63.40</td>
</tr>
<tr>
<td></td>
<td>Minimum 0.0</td>
<td>Minimum 0.0</td>
</tr>
<tr>
<td></td>
<td>Maximum 249.9</td>
<td>Maximum 246.9</td>
</tr>
</tbody>
</table>
Figure 4.1: Mean H3N8 antibody level of weanlings, yearlings, broodmares, teasers, horses in training and non-Thoroughbreds

Numbers above the antibody level indicate significant difference between the various groups. For example, 2, 3, 4, 5, 6* indicates that the mean H3N8 antibody level of weanlings is significantly different from that of the yearlings, broodmares, teasers, horses in training and non-Thoroughbreds (p < 0.01).
Figure 4.2: Mean H7N7 antibody level of weanlings, yearlings, broodmares, teasers, horses in training and non-Thoroughbreds

Numbers above the antibody level indicate significant differences between the various groups. For example, 2, 3, 5* indicates that the mean H7N7 antibody level of weanlings is significantly different from that of the yearlings, broodmares and horses in training (p <0.01).
Table 4.2: Distribution of EI H3N8 SRH levels in selected populations

<table>
<thead>
<tr>
<th>SRH</th>
<th>Weanling</th>
<th>Yearling</th>
<th>Broodmare</th>
<th>Teaser</th>
<th>Horses in Training</th>
<th>Non-TB</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50mm²</td>
<td>183 (91.5%)</td>
<td>74 (34.4%)</td>
<td>7 (3.4%)</td>
<td>28 (40.0%)</td>
<td>18 (8.1%)</td>
<td>77 (32.6%)</td>
<td>387 (33.7%)</td>
</tr>
<tr>
<td>&gt;50mm²</td>
<td>4 (2.0%)</td>
<td>26 (12.1%)</td>
<td>20 (9.8%)</td>
<td>14 (20.0%)</td>
<td>20 (9.0%)</td>
<td>39 (16.5%)</td>
<td>123 (10.7%)</td>
</tr>
<tr>
<td>&lt;85mm²</td>
<td>8 (4.0%)</td>
<td>58 (27.0%)</td>
<td>82 (40.0%)</td>
<td>24 (34.3%)</td>
<td>123 (55.2%)</td>
<td>78 (33.1%)</td>
<td>373 (32.5%)</td>
</tr>
<tr>
<td>&gt;85mm²</td>
<td>5 (2.5%)</td>
<td>57 (26.5%)</td>
<td>96 (46.8%)</td>
<td>4 (5.7%)</td>
<td>62 (27.8%)</td>
<td>42 (17.8%)</td>
<td>266 (23.2%)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>215</td>
<td>205</td>
<td>70</td>
<td>223</td>
<td>236</td>
<td>1149</td>
</tr>
</tbody>
</table>

Table 4.3: Distribution of EI H7N7 SRH levels in selected populations

<table>
<thead>
<tr>
<th>SRH</th>
<th>Weanling</th>
<th>Yearling</th>
<th>Broodmare</th>
<th>Teaser</th>
<th>Horses in Training</th>
<th>Non-TB</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50mm²</td>
<td>177 (88.5%)</td>
<td>86 (40.0%)</td>
<td>8 (3.9%)</td>
<td>56 (80.0%)</td>
<td>30 (13.5%)</td>
<td>186 (78.8%)</td>
<td>543 (47.3%)</td>
</tr>
<tr>
<td>&gt;50mm²</td>
<td>11 (5.5%)</td>
<td>13 (6.0%)</td>
<td>5 (2.4%)</td>
<td>1 (1.4%)</td>
<td>3 (1.3%)</td>
<td>10 (4.2%)</td>
<td>43 (3.7%)</td>
</tr>
<tr>
<td>&lt;85mm²</td>
<td>4 (2.0%)</td>
<td>45 (20.9%)</td>
<td>55 (26.8%)</td>
<td>5 (7.1 %)</td>
<td>70 (31.4%)</td>
<td>18 (7.6%)</td>
<td>197 (17.1%)</td>
</tr>
<tr>
<td>&gt;85mm²</td>
<td>8 (4.0%)</td>
<td>71 (33.0%)</td>
<td>137 (66.8%)</td>
<td>8 (11.4%)</td>
<td>120 (53.8%)</td>
<td>22 (9.3%)</td>
<td>366 (31.9%)</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>215</td>
<td>205</td>
<td>70</td>
<td>223</td>
<td>236</td>
<td>1149</td>
</tr>
</tbody>
</table>
Table 4.4a: Distribution of EI H3N8 and H7N7 SRH levels on weanling, yearling and broodmare premises (≥1 of 5 horses tested on each premises)

<table>
<thead>
<tr>
<th>SRH</th>
<th>Weanling Premises n=40</th>
<th>Yearling Premises n=43</th>
<th>Broodmare Premises n=41</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3N8</td>
<td>H7N7</td>
<td>H3N8</td>
</tr>
<tr>
<td>&lt;50mm²</td>
<td>40 (100.0%)</td>
<td>39 (97.5%)</td>
<td>29 (67.4%)</td>
</tr>
<tr>
<td>&gt;50mm²</td>
<td>8 (20.0%)</td>
<td>14 (35.0%)</td>
<td>38 (88.4%)</td>
</tr>
<tr>
<td>&gt;85mm²</td>
<td>7 (17.5%)</td>
<td>7 (17.5%)</td>
<td>36 (83.7%)</td>
</tr>
<tr>
<td>&gt;150mm²</td>
<td>3 (7.5%)</td>
<td>6 (15.0%)</td>
<td>24 (55.8%)</td>
</tr>
</tbody>
</table>

Table 4.4b: Distribution of EI H3N8 and H7N7 SRH levels on weanling, yearling and broodmare premises (all horses (5) tested on each premises)

<table>
<thead>
<tr>
<th>SRH</th>
<th>Weanling Premises n=40</th>
<th>Yearling Premises n=43</th>
<th>Broodmare Premises n=41</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3N8</td>
<td>H7N7</td>
<td>H3N8</td>
</tr>
<tr>
<td>&lt;50mm²</td>
<td>31 (77.5%)</td>
<td>26 (65.0%)</td>
<td>5 (11.6%)</td>
</tr>
<tr>
<td>&gt;50mm²</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>14 (32.6%)</td>
</tr>
<tr>
<td>&gt;85mm²</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>10 (23.3%)</td>
</tr>
<tr>
<td>&gt;150mm²</td>
<td>0 (0.0%)</td>
<td>0 (0.0%)</td>
<td>2 (4.7%)</td>
</tr>
</tbody>
</table>

4.4.1 TB weanlings: One hundred and eighty three of the 200 weanlings had an H3N8 antibody level of <50mm². On 31 (77.5%) of the 40 premises, all five weanlings had an H3N8 antibody measurement of <50mm² thus all of the horses tested on these premises were potential index cases (Table 4.4b). On 15 of the 31 premises all 5 weanlings were seronegative for H3N8. On 13 of these premises the weanlings tested were also seronegative for H7N7.

Only five (2.5%) of the 200 weanlings were virologically protected against H3N8 and these five weanlings were located on four different premises. Eight (4.0%) weanlings were clinically protected and four (2.0%) were partially protected against H3N8 (Figure 4.3). Antibodies to the H7N7 virus were detected in 23 (11.5%) weanlings on 14 premises. Ten of the 17 weanlings that had H3N8 antibody levels of >50mm² also had H7N7 antibody levels of >50mm².
Figure 4.3: Level of H3N8 Antibody Protection in Weanlings

4.4.2 TB yearlings: Over one third of the yearlings i.e. 74 (34.4%) had index case potential against H3N8. In five of the 43 premises all five yearlings had an antibody measurement of <50mm\\(^2\) and in two of these premises all five yearlings sampled were seronegative (Table 4.4b). Over half of the 215 yearlings tested had either virological or clinical protection against H3N8. Antibody levels indicative of virological protection were detected in 57 (26.5%) of the 215 yearlings in 24 (55.8%) of the 43 premises. Fifty-eight (27.0%) of the yearlings on 29 of the premises were clinically protected. Of these 29 premises 17 had yearlings that were both virologically and clinically protected. Twenty-six (12.1%) of the 215 yearlings were partially protected (Figure 4.4).

Antibodies against the H7N7 virus were detected in 131 of the 215 yearlings (60.9%) at the time of sampling. In 14 (32.6%) of the 43 premises all five yearlings had measurable antibody levels to H7N7 (Table 4.4b). In 11 (25.6%) of the 43 premises four of the five yearlings had measurable antibody levels. In four (9.3%) premises three of the five animals had measurable antibodies to H7N7 while in two (4.7%) premises two of the five animals sampled had measurable antibodies. In one single (2.3%)
premises only one of the five yearlings had detectable antibodies to H7N7. In 11 (25.6%) of the 43 premises all five yearlings sampled were seronegative for H7N7. On one of these premises all five yearlings were either virologically or clinically protected against H3N8.

![Yearlings (n=215)](image)

**Figure 4.4: Level of H3N8 Antibody Protection in Yearlings**

4.4.3 **TB broodmares:** Only seven (3.4%) of the 205 broodmares sampled had index case potential and five of these were seronegative for H3N8. There were no premises sampled during the course of this study where all five broodmares were potential index cases (Table 4.4b). The majority of the broodmares were either virologically or clinically protected. H3N8 antibody levels indicative of virological protection were detected in 96 (46.8%) of the 205 broodmares in 38 of the 41 premises. Eighty-two (40.0%) of the 205 broodmares were clinically protected while 20 (9.8%) of the mares were partially protected against the virus (Figure 4.5).

Antibodies to the H7N7 virus were detected in 197 (96.1%) of the 205 broodmares at the time of sampling. Of the eight broodmares that did not have measurable antibodies
to H7N7, two had clinical protection against H3N8 while four of the eight were partially protected against the H3N8 virus. The remaining two mares that were seronegative to H7N7 did not have measurable antibodies to EIV H3N8.

**Figure 4.5: Level of H3N8 Antibody Protection in Broodmares**

**4.4.4 Teaser Stallion population:** Twenty eight (40%) of the 70 animals sampled in this category had index case potential. Twenty one (30%) of these were seronegative. Only four (5.7%) animals in this group were virologically protected, 24 (34.3%) were clinically protected and 14 (20%) had partial protection (Figure 4.5).

Fifty six (80%) teasers were seronegative for H7N7 of which two horses had virological protection against H3N8. Thirteen of these horses had clinical protection against H3N8 while 13 were partially protected against the virus. Of the remaining 28 horses that were seronegative to H7N7, 21 were also seronegative for H3N8 and the remainder had index case potential.
4.4.5 Thoroughbred horses in training: Eighteen (8.1%) of the 223 horses in training had index case potential and six of these were seronegative. Sixty two horses (27.8%) were virologically protected, 123 (55.2%) were clinically protected and 20 (9.0%) were partially protected (Figure 4.7).

Antibodies to the H7N7 virus were detected in 196 (87.9%) of the 223 horses. Of the 27 horses that did not have measurable antibodies to H7N7, one horses had virological protection against H3N8, 11 horses had clinical protection and five horses were partially protected. Of the remaining 10 horses that were seronegative to H7N7 five were also seronegative to H3N8 and the remainder had index case potential.
4.4.6 **Non-Thoroughbred population:** Seventy seven (32.6%) of the 236 horses included in this section had index case potential and 66 of these were seronegative. Forty two (17.8%) had virological protection, 78 (33.1%) were clinically protected and 39 (16.5%) were partially protected (Figure 4.8).

Antibodies to EIV H7N7 were detected in 51 (21.6%) of the 236 horses. Of the 185 (78.4%) horses that did not have measurable antibodies to H7N7, 15 horses had virological protection against H3N8, 57 horses had clinical protection and 37 horses were partially protected. Of the remaining 76 horses that were seronegative to H7N7 67 were seronegative for H3N8 and the remainder had index case potential.
Figure 4.8: Level of H3N8 Antibody Protection in non-Thoroughbreds

### 4.5 Discussion

The horse industry in Ireland is broadly subdivided into the Thoroughbred or racing, and the non-Thoroughbred sports and pleasure horse sectors. The precise number of horses is unknown however in 2008, 12,419 Thoroughbred foals were registered as born in Ireland and at that time there were approximately the same number of registered Thoroughbred yearlings (HRI, 2008). There were 20,038 registered Thoroughbred broodmares and over 12,000 horses returned in training (HRI, 2008). In addition, it was estimated that there were over 110,000 non-thoroughbred horses (HSI, 2008). Horses from each of these categories were included in this study to determine the sectors of the industry that are most at risk from influenza. Ireland has an active EI surveillance programme (funded by the Department of Agriculture) that serves as an early warning system for the entire industry. The highly contagious nature of the virus and the level of susceptibility of the horse population contribute to the dissemination of virus and the spread of disease. It is estimated that one infected horse is capable on average, of infecting 10 susceptible, in-contact horses (Glass et al., 2002). The
identification of susceptible populations facilitates targeted vaccination to reduce the impact of an influenza outbreak and disruption of equestrian events.

Results obtained in a study carried out by Davies and Grilli (1989) in the U.K., which examined the susceptibility of humans to influenza virus, indicated that the infection rate for those with no detectable antibodies against the virus was 80%. In the equine population horses with SRH antibody levels of <50mm$^2$ are at greatest risk from influenza virus (Wood, 1991). Data from experimental challenge studies and observations in the field suggest that horses with SRH antibody levels of > 85mm$^2$ are likely to be protected against clinical disease and that those with levels of 150mm$^2$ or greater are likely to be protected against infection and virus shedding (Mumford et al., 1994; Newton et al., 2000a; Mumford, 2001). Thus in this study SRH antibodies against H3N8 were considered as a correlate of protection. Antibodies against H7N7 were considered as indicative of vaccination as this subtype is believed to be extinct (Webster et al., 1993) but is contained in four of the five vaccines on the Irish market. To our knowledge, this is the first study to compare SRH antibody levels of these different equine populations within a country although young Thoroughbred racehorses have been investigated in Newmarket (Newton et al., 2000b).

Results of this study indicate that the order of susceptibility to EI in the populations examined in Ireland was as follows: Thoroughbred weanlings > Teasers > non-Thoroughbred horses and ponies > Thoroughbred yearlings > Thoroughbred horses in training > Thoroughbred broodmares, with the Thoroughbred weanlings being the most susceptible and the Thoroughbred broodmares being the least susceptible to EIV infection. The fact that the H3N8 antibody levels of the weanlings, yearlings, broodmares and horses in training were similar to their H7N7 antibody levels suggests that their antibodies were primarily vaccinal in origin. This is consistent with the mandatory vaccination programme for racehorses implemented by the Turf Club and the vaccination requirements for some Thoroughbred sales. The teasers and non-Thoroughbreds had significantly higher H3N8 antibody levels than H7N7 antibody levels suggesting that the majority of the horses that were seropositive in these populations had been exposed to H3N8 by natural infection. The H3N8 antibody levels of horses in the study that were seropositive to both subtypes correlated significantly
with their H7N7 antibody levels i.e. there was no evidence of increased antibody response to H3N8 as a result of natural infection in vaccinated horses.

It is estimated that the weanlings included in this study were 6 to 10 months of age. Maternal antibodies against EI usually decline within six months (van Oirschot et al., 1991; van Maanen et al., 1992; Cullinane et al., 2001). Vaccination of foals before the maternal antibodies have waned may be of benefit if using a canarypox recombinant vaccine (Minke et al., 2007) but is not generally recommended if using conventional inactivated or subunit vaccines (van Maanen et al., 1992; Cullinane et al., 2001; Wilson et al., 2001). In Ireland most breeders do not commence vaccination until the foals are more than eight months of age. Over 70% and 88% of weanlings in this study were seronegative for H3N8 and H7N7 respectively and on 13 of the 40 premises examined, all of the weanlings tested were seronegative for both subtypes. This suggests that the majority of the weanlings had no detectable maternal antibodies and had not commenced their vaccination programme. It was not possible to determine whether some of the low levels of antibody detected against H3N8 and H7N7 were residual maternal antibodies or a response to first vaccination.

Over 65% of the yearlings in this study had SRH antibody levels against H3N8 of >50mm². Their mean SRH antibody level was 94 mm² which was higher than that recorded by Newton et al (2000b) in 222 yearlings entering training in Newmarket (64 mm²). It is recommended that EI vaccination commences after maternal antibodies have waned (Cullinane et al., 2001) and the sales companies in Ireland require that yearlings entering the autumn sales have up to date vaccination records and have at least completed the primary course of two vaccinations. The SRH levels against H7N7 suggested that the majority (>60%) of the yearlings had been vaccinated with one of the four vaccines containing this subtype and on one of the 43 premises the high H3N8 antibody levels and absence of H7N7 antibodies were consistent with vaccination with the canarypox recombinant vaccine or exposure by natural infection.

Although the yearlings in this study had a much lower index case potential than the weanlings (34% compared to 92%) over a third of them had low SRH levels suggesting a high susceptibility to EI. Vaccination in accordance with Turf Club Rules and the vaccine manufacturer’s recommendations can frequently leave horses with low
antibody titres for several months between their second and third vaccination (Newton et al., 2000a; Cullinane et al., 2001). Therefore, the time of year at which the primary course is administered and the timing and frequency of subsequent booster vaccinations might explain why some vaccinated animals do not have protective antibody levels. The existence of poor responders to vaccination is also well recognized and these horses play an important role in the amplification of virus and the spread of infection (Mumford, 2001). Vaccination records in the official passports of yearlings included in a study carried out by Newton et al., (2000b) indicated that 23% of them were not previously vaccinated. In this study 54 of the 215 yearlings (25%) were seronegative to both H3N8 and H7N7 suggesting that they were either poor responders or had not been vaccinated. This included three premises where all the yearlings tested were seronegative.

The Turf Club requires that racehorses receive two primary vaccinations administered 21 to 92 days apart, followed by a third vaccination administered 150 to 215 days after the second dose and annual vaccination thereafter. In this study the horses in training had higher antibody levels than the weanlings or the yearlings. The mean SRH level of 121mm$^2$ was similar to the level of 115mm$^2$ reported for two year olds in training in Newmarket (Newton et al., 2000b). Young horses are particularly susceptible to EI due to mixing at training facilities and racetracks and possibly, the impact of training regimes on the immune system (Folsom et al., 2001). The 8% identified as having index case potential in this study, particularly the five seronegative horses, represent a risk to their cohorts. Potential explanations for their poor EI immune status include the possibility that they responded poorly to vaccination or that they had only recently entered the training yard and had not been vaccinated.

In order to ensure a good supply of colostral antibodies for the foal, brood mares should be vaccinated in the latter stages of pregnancy but not later than 2 weeks prior to foaling (Cullinane et al., 2001). However, there are no mandatory requirements for vaccination of this population in Ireland. Broodmares were the best protected population investigated in this study with a mean SRH level of 143mm$^2$ and an index case potential of only 3.4%. Only two mares seronegative for H3N8 and H7N7 were identified in a population of 205. The majority of Thoroughbred broodmares would
have been vaccinated for several years while in training and they may also have been exposed to virus by natural infection at some time.

The majority of teasers are pony stallions. Because of the nature of their work they are exposed to a large number of broodmares during the breeding season. Forty per cent of the teasers included in this study had index case potential and 21 of 70 were seronegative for both H3N8 and H7N7. Only 4 were virologically protected against H3N8. The mean antibody level for H3N8 and H7N7 was 66mm$^2$ and 31mm$^2$ respectively. The results strongly suggest that the teaser population is inadequately vaccinated and could play a major role in transmission to susceptible broodmares during an outbreak.

The level of susceptibility observed in the non-Thoroughbred population was similar to that observed in the Thoroughbred yearling population in that approximately half of the population was either virologically or clinically protected while the other half was only partially protected or had index case potential. Horses competing under the auspices of the FEI require EI vaccination within six months +21 days of the competition and many equine associations and clubs require vaccination. Vaccination is recommended for all horses in Ireland but vaccination is not mandatory for the majority of pleasure horses. The mean H3N8 and H7N7 antibody levels for the non-Thoroughbreds tested in this study were 84mm$^2$ and 30mm$^2$ respectively. Antibodies against H7N7, which are indicative of vaccination, were observed in only 21.6% of this population at the time of sampling. The results suggest many non-Thoroughbreds have antibodies as a result of natural infection but that the overall immunological status could be significantly improved by regular vaccination.

The circumstances surrounding the induction of antibodies may influence their correlation to protection against EI and the only really satisfactory way to determine whether horses are protected is to conduct virus challenge experiments. Such experiments are not practical with horses retained for competition, breeding and pleasure riding. The nature of the immune response to natural infection differs from that elicited by vaccination and in our study the potential vulnerability of some of the horses in for example, the non-Thoroughbred sector, may be over estimated. Resistance to re-infection has been documented to exist for one year and to persist even after levels
of antibody become barely detectable (Hannant et al., 1988). Similarly, the level of protective immunity of some of the vaccinated Thoroughbreds in this study could well prove to be overestimated if they were exposed to a virus strain that differed significantly from the vaccine strains. Higher SRH antibody levels are required to protect against a heterologous virus (Newton et al., 1999; Daly et al., 2004). These caveats aside, the results obtained from this study provide useful information on the potential susceptibility of different populations to EI.

This study identified weanlings, teasers and non-Thoroughbred horses as the populations most susceptible to EI on the basis of their SRH antibody levels. There is currently no mandatory EI vaccination for entry to weanling sales in Ireland. Although to date there is little epidemiological evidence to indicate that the weanling population is particularly susceptible to influenza, our results suggest that it would be advisable that weanlings have commenced their vaccination programme prior to attendance at public sales. Unvaccinated teaser stallions played a key role in the spread of EI on a public stud in Ireland in 2008 (Gildea et al, 2011b) and this study has identified the potential for this to occur on other farms. As a minimum, teaser stallions should be vaccinated against EI in accordance with the manufacturers’ instructions and preferably, should receive a booster before each breeding season. Outbreaks of EI in Ireland are usually diagnosed in the non-Thoroughbred population and in horses in training (Gildea et al, 2011b). During the 1989 EI epidemic in Ireland the disease was first identified in the non-Thoroughbreds and then spilled over into the racehorse population (van Maanen and Cullinane, 2002). Currently vaccination against EI is not required for entry to many of the non-Thoroughbred sales, fairs and local shows. The introduction of mandatory vaccination for participation in such events would reduce the risk of amplification of virus in this population and dissemination to other populations. Only through a combined approach of vaccination, meticulous surveillance and the evaluation of levels of immunological protection in our equine populations can we minimize disruption to training schedules and equestrian events.
Acknowledgements

The experimental work was carried out at the Irish Equine Centre and funded by the Department of Agriculture under the National Development Plan. The results will be submitted as part of a PhD thesis by Sarah Gildea to the University of Limerick.

4.6 References


which recently vaccinated horses in Newmarket developed respiratory disease', *Vet Rec*, 158(6), 185-92.


Wood, J. L. N. (1991) Equine Influenza: 'A Review of the History and Epidemiology and a Description of Recent Outbreak’. MSc Dissertation, London School of Hygiene and Tropical Medicine, University of London.
Chapter 5

A comparison of antibody responses to commercial equine influenza vaccines following annual booster vaccination of National Hunt Horses – a randomised blind study

Published in: Vaccine, 2011, 29(22) 3917-22.

Authors: Sarah Gildea, Cathal Walsh, Sean Arkins, Ann Cullinane
5.1 Abstract

Protection against equine influenza virus (EIV) relies largely on the production of circulating antibodies specific for the haemagglutinin (HA) glycoprotein. The objective of this study was to determine the antibody responses of National Hunt horses in training to booster vaccination. The antibody responses to the six equine influenza vaccines available in Ireland (three whole inactivated vaccines, two subunit vaccines and a canarypox recombinant vaccine) were monitored by single radial haemolysis (SRH) for six months post vaccination. There was no significant difference between the levels of antibody response induced following booster vaccination with any of the six vaccines. The antibody levels peaked between two and four weeks post vaccination, decreased significantly by three months post vaccination and declined to their original levels by six months post vaccination. Peak antibody responses to the canarypox recombinant vaccine were delayed in comparison to the other vaccines. Although analysis of the mean SRH levels of the horses suggested that they were clinically protected post booster vaccination, analysis of the individual responses suggested that there was potential for vaccination breakdown in a manner similar to that observed previously in racing yards in Ireland. There was a significant correlation between the SRH level at the time of vaccination and the subsequent antibody response. The findings of this study suggest that it would be advantageous to monitor SRH levels and to vaccinate strategically. The revaccination of horses with low antibody levels three months post booster vaccination may have been more effective in protecting horses in this yard than the annual vaccination of horses with high SRH levels. Eighteen of the 44 (41%) horses included in this study did not demonstrate a significant rise in SRH level to H3N8 following booster vaccination. It is presumed that annual revaccination is the minimum necessary to protect all horses against EI but this assumption needs to be systematically evaluated. It has been demonstrated that shorter intervals are required for optimum protection of young horses and it may be that longer vaccination intervals are sufficient for older horses with several years of vaccination history. Further investigations in a larger population of horses will be necessary to determine if the findings of this study are applicable to the population at large.
5.2 Introduction

Protection against equine influenza virus (EIV) relies largely on the production of circulating antibodies specific for the haemagglutinin (HA) glycoprotein. Antibodies against the HA are produced following exposure to the virus or following vaccination. Measurement of antibodies against the HA can be carried out using the haemagglutination inhibition (HI) or single radial haemolysis (SRH) test. However, a comparison of influenza serological techniques carried out in an international collaborative study demonstrated that the SRH test was the more sensitive and more reproducible between laboratories (Mumford, 2000; Daly et al., 2007). In addition, a definitive correlation between antibody levels measured by the SRH test and protective immunity against equine influenza (EI) H3N8 has been established in both experimental challenge studies and the field (Mumford and Wood, 1992; Mumford et al., 1994a; Newton et al., 2000a).

Vaccination of horses against EI substantially reduces the spread of infection as well as significantly reducing the severity and duration of clinical signs of the disease. Mandatory vaccination of racehorses was introduced in Ireland in 1981 and since its introduction thirty years ago, no race meetings have been cancelled as a result of EI. In Ireland the Turf Club requires that the first two doses of a primary vaccination course be administered between 21 and 92 days apart followed by a third vaccination 150 to 215 days from the second vaccination. Thereafter EI booster vaccinations must be administered annually, with the last permissible day being the same date as the previous year's vaccination. This schedule is similar to that of the Jockey Club in the United Kingdom and is in line with manufacturer’s guidelines (Newton et al., 2005).

The type of EI vaccines currently available in Ireland can be described as first and second-generation. First generation or inactivated vaccines include both traditional whole virus vaccines and more recent subunit vaccine preparations while the only second generation vaccine currently available on the Irish market is a canarypox virus recombinant vaccine. First generation vaccines traditionally contain both types of equine influenza subtype 1 (H7N7) and subtype 2 (H3N8) even though the H7N7 virus appears to be extinct (Webster, 1993) and the OIE no longer recommend that a virus of this subtype be included in the vaccines (Cullinane et al., 2010). The canarypox recombinant vaccine contains representative strains of H3N8 only.
Horses which fail to produce a durable protective response following vaccination, will inevitably assist in virus spread during an outbreak, which can have significant financial implications. Published data indicate that horses with SRH antibody levels of 150mm$^2$ or greater are protected against virus infection and that horses with SRH antibody levels of 85mm$^2$ or greater are clinically protected (Mumford and Wood, 1992; Mumford et al., 1994a; Mumford, 2001), provided that the vaccine strain and the outbreak strain are antigenically similar (Daly et al., 2004). The objective of this study was to determine the antibody responses of National Hunt horses in training to booster vaccination. Six EI vaccines were available in Ireland at the time of the study. The SRH antibody response to each of these products was monitored for six months post vaccination.

5.3 Materials and Methods

5.3.1 Horses
This study was carried out on a population of 44 Thoroughbred National Hunt horses in training on a single premises. The population size was dictated by reliance on the cooperation of a trainer to supply racehorses and agree to the necessary blood sampling regime. Horses of both genders were included in this study and they ranged in age from three to 11 years. The number of horses in each age group was as follows: 2 three-year-olds, 11 four-year-olds, 11 five-year-olds, 6 six-year-olds, 6 seven-year-olds, 3 eight-year-olds, 4 nine-year-olds and 1 eleven-year-old. The mean age was 5.8 ± 0.28 SEM years.

5.3.2 Vaccines
The type, adjuvant and composition of each of the six vaccines included in this study is shown in Table 5.1. The multivalent vaccine Equilis Resequin (Intervet) combines EI and equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4). In addition, Equilis Resequin was the only vaccine included in this study where a combined influenza/tetanus vaccine was not available. In the case of horses vaccinated with this product, tetanus toxoid (Intervet) was administered separately by the veterinary surgeon in accordance with the manufacturer’s guidelines.
Table 5.1: Vaccine product details

<table>
<thead>
<tr>
<th>Vaccine/Manufacturer</th>
<th>Nature</th>
<th>Adjuvant</th>
<th>Strain/Subtype/Antigenic Mass</th>
<th>Tet Tox</th>
<th>No. Horses</th>
</tr>
</thead>
</table>
| Prevac T Pro (Intervet) | Inact whole | Alum. Hydr. | A/eq/Prague/56 (H7N7) (50 µg)  
A/eq/Newmarket/2/93 (H3N8) (20 µg)  
A/eq/Newmarket/1/93 (H3N8) (20 µg) | 150 IU | 8 |
| Duvaxyn IE-T Plus (Fort Dodge) | Inact whole | Carbomer, Alum. Hydr. | A/eq/Prague/56 (H7N7) (15-18 µg)  
A/eq/Suffolk/89 (H3N8) (15-18 µg)  
A/eq/Newmarket/1/93 (H3N8) (15-18 µg) | 18 Lf | 7 |
| Equilis Resequin* (Intervet) | Inact whole | Alum. Hydr., Immunostim | A/eq/Prague/56 (H7N7) (50 µg)  
A/eq/Newmarket/2/93 (H3N8) (20 µg)  
A/eq/Newmarket/1/93 (H3N8) (20 µg) | N/A | 7 |
| Equilis Equenza T (Intervet) | Subunit vaccine | Quillaic Acid derivative | A/eq/Prague/56 (H7N7) (100 AU)  
A/eq/Newmarket/2/93 (H3N8) (50 AU)  
A/eq/Newmarket/1/93 (H3N8) (50 AU) | 40 Lf | 7 |
| Equip FT (Schering Plough) | Subunit vaccine | ISCOM, Quillaic Acid derivative, Aluminium phosphate | A/eq/Newmarket/77 (H7N7) (10 µg)  
A/eq/Kentucky/98 (H3N8) (1.4 µg)  
A/eq/Borlange/91 (H3N8) (20 µg) | 100Lf | 9 |
| ProteqFlu-Te (Merial) | CPRV | Carbomer | A/eq/Kentucky/94 (H3N8) (> 6.5 log 10 FAID 50)  
A/eq/Newmarket/2/93 (H3N8) (> 6.5 log 10 FAID 50) | >30 IU | 6 |

Tet Tox = Tetanus Toxoid, Inact whole = Inactivated whole, Alum Hydr = Aluminium Hydroxide IU = International Unit, Lf = Limes flocculation units, * = multivalent vaccine, N/A = not available, AU = antigenic units, CPRV = Canarypox recombinant vaccine; Recombinant vaccine, FAID 50 = Fluorescent assay infectious dose.
5.3.3 Vaccinations
Previous vaccination histories of horses included in this study were unknown but all horses had completed at least their primary course and were due their annual booster vaccination at the time of this study. Animals were stratified according to antibody levels prior to booster vaccination and allocated to one of the six vaccine groups using the random number generator available within Microsoft Excel. Gender or age of horse at the time of vaccination was not a factor in vaccine allocation. Each horse was given a single dose of vaccine by deep intramuscular injection according to the manufacturer’s instructions. The number of horses assigned to each vaccine group is shown in Table 5.1.

5.3.4 Collection of samples
Whole blood samples were collected from the horses at five different intervals as shown in Table 5.2. Samples were submitted to the laboratory following collection and serum was stored at -20°C until tested.

Table 5.2
Timetable for vaccination and collection of samples from horses

<table>
<thead>
<tr>
<th>Weeks</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>12</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccination</td>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling</td>
<td>S1</td>
<td>S2</td>
<td>S3</td>
<td>S4</td>
<td>S5</td>
</tr>
</tbody>
</table>

5.3.5 Serology
Antibodies against A/eq/Newmarket/2/93 (H3N8) a representative of the Eurasian lineage, A/eq/Kildare/92 (H3N8) a representative of the American lineage, A/eq/South Africa/4/03 (H3N8) a representative of the Florida sub-lineage of the American lineage and A/eq/Prague/56, the prototype H7N7 virus, were measured using the SRH test according to standard procedures (OIE, 2008). In brief, optimized viral antigens were coupled to sheep red blood cells (SRBC) with chromium chloride. Agarose plates (1% wt/vol) were prepared containing the sensitized SRBC’s and guinea pig complement.
The test sera were heat inactivated at 56 °C ± 1 °C for 30 minutes, 10 µl was aliquoted into wells on the plate and the plates were incubated at 34 °C ± 1 °C for 20 to 24 hours.

Control antisera against A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/2/93 (H3N8), A/eq/Newmarket/1/93 (H3N8) and A/eq/South Africa/4/03 (H3N8) from the European Directorate for the Quality of Medicines and Healthcare (EDQM), were included on each plate as appropriate. The haemolytic zones resulting from the lysis of the antigen-coated SRBC’s by the antibody in the test sera were measured with a viewer and digital recording apparatus (Mitutoya, Aurora, Illinois, USA). The area of haemolysis was calculated and results were expressed in mm². Mean H3N8 antibody values were calculated from SRH results obtained against the H3N8 antigens A/eq/Kildare/92, A/eq/Newmarket/2/93 and A/eq/South Africa/4/03. A significant rise in titre was defined as an increase in the SRH level of 25 mm² or 50% whichever is smaller between paired serum samples (Newton et al., 2000a). The laboratory investigator was blinded to vaccine allocation to individual horses. The responses to tetanus toxoid and equine herpes virus (Equilis Resequin only) following vaccination were not examined during this study.

5.3.6 Statistical analysis
All statistical analysis was carried out on the open source package R version 2.8.1 (The R Foundation for Statistical Computing, Austria). Data were analysed using a repeated measures analysis of variance and post hoc testing was carried out with Tukey’s Honest Significant Difference (HSD). The area under the curve (AUC) as described by Heldens et al., (2002a) was calculated by the trapezoidal rule and used as the metric for the repeated measures analysis of SRH antibody levels. The affect on antibody levels over time following booster vaccination with each of the six vaccines was also investigated.
5.4 Results

5.4.1 Course of antibody response

The pattern of antibody responses was similar for all vaccines (Figures 5.1–5.4). Highest antibody levels against all antigens except A/eq/Kildare/92 (H3N8) were recorded two weeks post vaccination with five of the six vaccines and highest antibody levels against all H3N8 antigens were recorded four weeks post vaccination with the sixth vaccine (ProteqFlu Te). There was a significant decrease in antibody levels against the representative of the Eurasian lineage A/eq/Newmarket/2/93 (H3N8), between two weeks and one-month post vaccination with all vaccines except ProteqFlu Te (P ≤0.01). There was also a significant decrease in antibody levels against A/eq/Kildare/92 (H3N8) and A/eq/South Africa/4/03 (H3N8) with all vaccines between one and three months post vaccination (P ≤0.001). The antibody levels against A/eq/Newmarket/2/93 (H3N8) decreased significantly between two weeks and three months post vaccination with all vaccines except ProteqFlu Te (P ≤0.001). The decrease in antibody levels against the H3N8 viruses between three and six months post vaccination was not significant. There was a significant decrease in antibody levels against A/eq/Prague/56 (H7N7) between two weeks and three months post vaccination with the five vaccines containing a H7N7 representative (P ≤0.001).

5.4.2 Comparison of antibody response

Results of a Tukey’s HSD comparison indicated that there was no significant difference between antibody responses induced following booster vaccination with any of the six vaccines included in this study Pr = 0.9336, 0.8897, 0.9905 and 0.974 for A/eq/Prague/56 (H7N7), A/eq/Kildare/92 (H3N8), A/eq/Newmarket/2/93 (H3N8) and A/eq/South Africa/04/03 (H3N8), respectively. Area under the curve (AUC) against the SRH antigens tested was also calculated for each of the six vaccines. A Tukey’s multiple comparisons of mean AUC indicated that there was no significant difference between vaccines over the different time points, Pr (>F) = 0.7977, 0.9221, 0.7725 and 0.6345 for A/eq/Prague/56, A/eq/Kildare/92, A/eq/Newmarket/2/93 and A/eq/South Africa/4/03, respectively.
Figure 5.1: SRH antibody response (mm$^2$) to A/eq/Prague/56 in the weeks following booster vaccination (Data are means +/- SEM)
Figure 5.2: SRH antibody response (mm²) to A/eq/Kildare/92 in the weeks following booster vaccination (Data are means +/- SEM)
Figure 5.3: SRH antibody response (mm$^2$) to A/eq/Newmarket/2/93 in the weeks following booster vaccination (Data are means +/- SEM)
Figure 5.4: SRH antibody response (mm$^2$) to A/eq/South Africa/4/03 in the weeks following booster vaccination (Data are means +/- SEM)
5.4.3 Failure or delay in H3N8 response to vaccination

Eighteen of the 44 (41%) horses included in this study did not exhibit an SRH increase of 25mm$^2$ or greater to H3N8 following booster vaccination and two had no detectable increase in antibody levels. A delay in response to vaccination was also observed in a horse vaccinated with ProteqFlu Te. This horse failed to respond by two weeks post vaccination but had responded by the time of S3 (four weeks post vaccination). Results of a Chi squared test indicate that a significantly higher proportion of horses with low SRH H3N8 antibody levels seroconverted two weeks post booster vaccination ($P \leq 0.001$) in comparison to those horses with high SRH H3N8 antibody levels at the time of booster vaccination. Results of this analysis are shown in Table 5.3 and illustrated in Figure 5.5.

Table 5.3: Influence of pre-existing H3N8 antibody levels on booster vaccination response

<table>
<thead>
<tr>
<th>Existing SRH H3N8 levels at time of V</th>
<th>Increase in SRH level of ≥25mm$^2$</th>
<th>Increase in SRH level of &lt;25mm$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;50mm$^2$ (Index case potential)</td>
<td>3/3 (100%)</td>
<td>0/3 (0%)</td>
</tr>
<tr>
<td>&gt;50mm$^2$ &lt;85mm$^2$ (partial protection)</td>
<td>6/6 (100%)</td>
<td>0/6 (0%)</td>
</tr>
<tr>
<td>&gt;85mm$^2$ &lt;150 mm$^2$ (Clinical protection)</td>
<td>14/21 (67%)</td>
<td>7/21 (33%)</td>
</tr>
<tr>
<td>&gt;150 mm$^2$ (Virological protection)</td>
<td>3/14 (21%)</td>
<td>11/14 (79%)</td>
</tr>
<tr>
<td>Total</td>
<td>26/44 (59%)</td>
<td>18/44 (41%)</td>
</tr>
</tbody>
</table>
Figure 5.5: The relationship between the SRH antibody levels on the day of vaccination (DV) and response to booster vaccination

5.4.4 Age of horse

The effect of animal’s age at the time of booster vaccination on vaccination response was examined using repeated measure analysis. Results obtained indicated that age was not an influencing factor on vaccine response: Pr (>F) = 0.1956, 0.9353, 0.692 and 0.7934 for A/eq/Prague/56 (H7N7), A/eq/Kildare/92 (H3N8), A/eq/Newmarket/2/93 (H3N8) and A/eq/South Africa/04/03 (H3N8), respectively.
5.5 Discussion

This study examined the serological responses of National Hunt horses in a training yard to annual booster vaccination against EI. The previous vaccination histories of the horses were unknown but all horses had at least received their primary course of vaccination. The influence of time since previous vaccination and total number of previous vaccines administered, on serum antibody levels were shown not to be significant for horses in training following administration of one or two doses of vaccine (Newton et al., 2000b). All of the EI vaccines available in Ireland were included in the study. In all cases tetanus toxoid was administered at the same time as EI either as a combined vaccine or, if unavailable, as an additional vaccine. Results of a study by Mumford et al., (1994a) indicated that the presence of tetanus toxoid did not compromise the response to EI vaccination. Blood samples taken prior to and after vaccination were tested for antibodies against EI by SRH. Although partial clinical protection has been demonstrated to persist for one year post infection in ponies with low levels of antibody, the immunity induced by vaccination with inactivated antigens correlates strongly with levels of SRH antibody (Hannant et al., 1988). Prior to booster vaccination, 14 (32%), 21 (48%), 6 (14%) and 3 (7%) of the 44 horses included in this study had virological protection, clinical protection, partial protection and index case potential respectively. There was no significant difference in antibody levels between the six groups assigned different vaccines and the mean H3N8 antibody level of 123.40 ± 6.458 mm$^2$ was similar to the antibody levels reported previously for horses in Irish racing yards (121mm$^2$) (Gildea et al., 2010). This antibody level is considered to afford clinical protection (Mumford and Wood, 1992; Mumford et al., 1994a; Mumford, 2011) if the challenge virus is closely related antigenically to the virus in the vaccine (Daly et al., 2004).

The pattern of humoral antibody responses was similar for all vaccines and for all antigens tested. The antibody levels peaked between two and four weeks post vaccination, (170.84±5.22 mm$^2$ - 160.38±4.10 mm$^2$) decreased significantly (P <0.01) by three months post vaccination (132.14±5.04 mm$^2$) and declined to their original levels by six months post vaccination (122.45±5.07 mm$^2$). The peak antibody levels for two of the three H3N8 antigens tested were recorded two weeks post vaccination for horses vaccinated with the whole inactivated vaccines (Prevac T Pro, Duvaxyn IET
Plus and Equilis Resequin) and the subunit vaccines (Equilis Equenza and Equip FT). The peak antibody levels for all H3N8 antigens tested were recorded four weeks post vaccination for five of the six horses vaccinated with the canarypox recombinant vaccine (ProteqFlu Te). One of these five horses failed to respond by two weeks but responded by four weeks post vaccination. It has been suggested that booster vaccination with the canarypox recombinant vaccine should be administered no later than two weeks prior to an event (Minke et al., 2007). Our data support this recommendation but suggest on the basis of the antibody responses, that it would be preferable to time booster vaccination with this type of vaccine, no later than four weeks prior to an event.

In this study all the horses that responded to vaccination experienced a decline in antibody levels within three months of vaccination. The rapid decline in the humoral response to EI vaccination in both naïve and primed equidae is well recognised (Mumford et al., 1994a). Experimental studies in pony foals demonstrated a decline in antibody levels after their third dose of vaccine with an ISCOM based subunit vaccine (Mumford et al., 1994a), a carbomer adjuvanted whole inactivated vaccine (Mumford et al., 1994b) and a canarypox recombinant vaccine (Minke et al., 2007). A study in seronegative Thoroughbred horses (4 to 12 months of age) vaccinated with an inactivated equine influenza–herpesvirus combination vaccine also demonstrated a rapid decline in antibody levels after third vaccination (Heldens et al., 2002b). Limited field data indicate a similar pattern in Thoroughbred yearlings which are particularly susceptible to influenza infection (Newton et al., 2000a). To our knowledge, no peer reviewed published data exist in relation to the durability of response to booster vaccination in older racehorses.

Although a decline in antibody levels was observed within three months of booster vaccination, at the time of the last sampling i.e. six months post vaccination, thirty nine of forty four (89%) horses had SRH antibody levels consistent with clinical protection i.e. similar to those at the time of booster vaccination. No significant difference in antibody responses existed amongst horses in each of the six vaccine groups included in this study. Neither local antibody responses nor cell mediated immunity (CMI) were measured during this study but may be relevant to some of the vaccines used. In virus challenge studies it has been suggested that CMI or local antibody responses reduced
clinical signs and virus shedding in a few ponies with low antibody levels post vaccination with an ISCOM based vaccine (Mumford et al., 1994a; Paillot et al., 2008). CMI has been demonstrated in humans after ISCOM vaccination (Rimmelzwaan et al., 2000) and EIV specific IFN-γ synthesis by peripheral blood lymphocytes has been described in ponies vaccinated with EQUIP F (Paillot et al., 2008). The canarypox vaccine presents the influenza haemagglutinin to the horse’s immune system in a way that mimics natural infection and may stimulate a greater CMI response than conventional vaccines (Toulemonde et al., 2005). Increased IFN-γ protein synthesis has been demonstrated following challenge infection with EIV in ponies vaccinated with ProteqFlu compared with control ponies (Paillot et al., 2006).

Although analysis of the mean SRH levels of the horses suggested that they were clinically protected post booster vaccination, analysis of the individual responses suggested that there was potential for vaccine breakdown in a manner similar to that observed in two racing yards in Ireland in 2007 (Gildea et al., 2011). Only seven of the 44 horses in this study (16%) had SRH antibody levels consistent with virological protection six months post vaccination. Thus the majority of horses in this yard had the potential to be infected and to shed virus. Six months post vaccination five horses had SRH levels below 85mm$^2$ and one horse was seronegative. Seronegative horses are 15 times more likely to be the index case in the event of an outbreak and have the potential to amplify virus and serve as a source of infection to partially protected cohorts (Wood, 1991). Two seronegative horses were the index cases in one of the vaccinated racing yards in 2007 where 29% horses exhibited clinical signs (Gildea et al., 2011).

In this study there was a significant association between the SRH levels at the time of vaccination and subsequent antibody responses. The horse that was the potential index case six months post vaccination had an SRH level of 30mm$^2$ at the time of vaccination. Although it mounted an excellent response to vaccination i.e. it had SRH levels of 139mm$^2$ and 116mm$^2$ two weeks and one month post vaccination respectively, the antibodies had declined to an undetectable level three months post vaccination. Furthermore, eighteen of the 44 (41%) horses included in this study did not demonstrate an SRH increase of 25mm$^2$ or greater to H3N8 following booster vaccination. All of these eighteen had SRH levels of >90mm$^2$ prior to vaccination. At the time of booster vaccination the mean H7N7 antibody levels of horses vaccinated
with ProteqFlu Te which does not contain an H7N7 virus, was 179mm$^2$ and the mean H7N7 antibody levels of horses vaccinated with the other vaccines containing both subtypes was 139mm$^2$. Between two and ten weeks post vaccination the mean H7N7 antibody levels of the ProteqFlu Te group was lower than the mean H7N7 antibody levels of horses vaccinated with the other five vaccines but between ten weeks and six months post booster vaccination their mean H7N7 antibody levels was similar. Thus on comparison of horses that received a booster vaccine with and without H7N7, the advantage derived from vaccination against this antigen in the presence of high SRH levels appeared to be short lived and the benefit of booster vaccination questionable. It is presumed that annual revaccination is the minimum necessary to protect all horses against EI but this assumption is neither evidence-based nor has it has been systematically evaluated. It has been demonstrated that shorter intervals are required for optimum protection of young horses (Cullinane et al., 2001; Park et al., 2003; Newton et al., 2000b), and it may be that longer vaccination intervals are sufficient for older horses with several years’ vaccination history.

In conclusion, this study suggests that it would be advantageous to monitor SRH levels and to vaccinate strategically. The revaccination of the potential index case three months post booster vaccination may have been more effective in protecting the horses in this yard than the annual vaccination of horses with high SRH levels. However, annual booster vaccination is currently required by the racing authorities in several countries and this mandatory vaccination policy has served the industry well (Gildea et al., 2010). Further epidemiological and experimental data on the duration of immunity will need to be obtained if longer vaccination intervals are to be considered for older horses. One of the limitations of this study was the small number of animals in each vaccine group. Larger numbers from a cross section of stables will be required to determine if the findings of this study are applicable to the population at large. The scientific validity of annual revaccination of companion animals has been questioned in recent years and protection has been proven in challenge experiments several years post vaccination against canine distemper virus, canine parvovirus and canine adenovirus (Schultz et al., 2010; Larson et al., 2007). Current vaccination practices in horses need to be scrutinised to ensure that protection from immunisation develops early in life and is sustained for as long as possible without over-vaccination.
Acknowledgements

This study would not have been possible without the cooperation of the racehorse trainer to whom the authors are extremely grateful. All of the experimental work was funded by the Department of Agriculture under the National Development Plan and carried out at the Irish Equine Centre. The results will be submitted as part of a PhD thesis by Sarah Gildea to the University of Limerick.

5.6 References


populations in Ireland to equine influenza virus', *Influenza Other Respi Viruses*, 4(6), 363-72.


Chapter 6

A comparison of antibody responses to commercial equine influenza vaccines following primary vaccination of Thoroughbred weanlings – a randomised blind study

Submitted to: Accepted for publication by: Vaccine

Authors: Sarah Gildea, Cathal Walsh, Sean Arkins, Ann Cullinane
6.1 Abstract

Many racing authorities, sales companies and equestrian bodies have mandatory vaccination policies for equine influenza (EI). The consequences of lack of vaccine efficacy include clinical disease, disruption to training programmes, the cancellation of equestrian events and the introduction of virus to susceptible populations. The correlation between levels of antibody against the virus haemagglutinin and protection against influenza has been well established. The objective of this study was to compare the antibody responses of 66 unvaccinated Thoroughbred weanlings on four different stud farms, following primary vaccination (V1, V2, V3) with the five EI vaccines commercially available in Ireland (Duvaxyn IET Plus, Equilis Resequin, Equip FT, Equilis Frequenza Te, ProteqFlu Te). Antibody responses were monitored for 26 weeks post V3 by single radial haemolysis. The pattern of antibody responses were similar for all vaccines and for all antigens tested. A rapid decline of antibody levels was observed by 13 weeks post V2 for all vaccines. The antibody responses of the horses vaccinated with the whole virus vaccine Duvaxyn IET Plus was significantly higher than those of the horses vaccinated with the other four products. Five weanlings had maternally-derived antibodies (MDA) at the time of V1. The canarypox recombinant vaccine, subunit vaccine and whole virus inactivated vaccines administered to these weanlings did not induce detectable antibody responses against the background of MDA but effectively primed the animals as revaccination resulted in strong antibody responses. In this study 43% of the weanlings failed to seroconvert after V1. This high incidence of poor responders has not been reported in previous experimental studies relating to these products. The poor responders were observed in all vaccine groups except those vaccinated with Duvaxyn IET Plus. Post V2 the incidence of poor responders was reduced to 7% and all horses responded to V3. The study demonstrates that independent evaluation of influenza vaccine performance in the field is critical to add to the body of knowledge gained from experimental challenge experiments carried out for regulatory or marketing purposes.
6.2 Introduction

Equine influenza virus (EIV), an orthomyxovirus, is a highly contagious respiratory pathogen of horses and other equidae. In countries where equine influenza (EI) is endemic the economic losses associated with outbreaks are minimised by vaccination. The majority of the vaccines contain representatives of the only two subtypes of influenza that are known to have adapted successfully to equidae i.e. H3N8 and H7N7. However, H7N7 viruses have not been isolated for over two decades and the World Organisation for Animal Health or OIE stipulates that there is no longer a requirement for a representative of this subtype in EI vaccines (OIE, 2010). An effective vaccine should prevent disease and virus shedding i.e. induce both clinical and virological protection. Protection against virus shedding has been shown to correlate with the degree of antigenic relatedness of the vaccine strain to the challenge virus (Daly et al., 2003). Mismatch between vaccine and infecting strains significantly increases the risk of an outbreak at the population level (Park et al., 2004). The consequences of lack of vaccine efficacy include clinical disease, disruption to training programmes, the cancellation of equestrian events and the introduction of virus to susceptible populations.

Vaccine efficacy is of importance to all countries irrespective of their disease status. Countries where EI is endemic rely on vaccination to minimise the incidence of disease and dissemination of viruses at competitions and other equestrian events. Vaccination failure at competitions has led not only to respiratory disease at show grounds but also to the wide geographical spread of virus when the competition is over and the horses return to their farms. Countries were EI is not endemic rely on vaccination of imported horses, in addition to quarantine, to prevent an incursion of the virus (Cullinane et al., 2010). Major outbreaks have occurred as a result of the international movement of breeding and competition horses vaccinated with vaccines that seem to have induced clinical but not virological protection i.e. transmission occurred as a result of subclinically infected vaccinated horses shedding virus. There is epidemiological evidence that influenza virus was introduced into South Africa (1986 and 2003), India (1987), Hong Kong (1992) and Australia (2007) with vaccinated horses from North America, Europe and Japan (Guthrie et al., 1999; King and MacDonald, 2004; Uppal et al., 1989; Powell et al., 1995; Garner et al., 2011). Some of these incursions had
devastating financial consequences for example, the control and eventual elimination of EI from Australia in 2007 is estimated to have cost one billion Australian dollars (Garner et al., 2011). Many racing authorities, sales companies and equestrian bodies have mandatory vaccination policies that assist in ensuring business continuity. In 1981 the Turf Club in Ireland, with the racing authorities in the United Kingdom and France, initiated mandatory vaccination following an epizootic of EI in Europe and North America that significantly impacted on the racing industry with reduced fields (number of horses entered for races) and cancelled meetings. The Irish Turf Club requires that race horses receive two primary vaccinations administered 21-92 days apart followed by a third vaccination administered 150-215 days after the second dose and annual vaccination thereafter (Turf Club, 2010). All horses participating in Federation Equestre Internationale (FEI) competitions must receive an initial primary course of three doses of vaccine, a minimum of annual vaccination and a booster dose within six months + 21 days of competition (FEI, 2011). The 21 day interval is provided to enable vaccination requirements to fit with the competition schedule.

As a result of mandatory vaccination programmes, trainers and owners represent a captive market for EI vaccines and need to be able to make informed decisions in relation to the product they use. The majority of vaccine evaluations are carried out in experimental ponies or occasionally horses, by vaccine companies or at the request of vaccine companies for submission to the regulatory authorities. Such studies tend to be conducted under optimal conditions for vaccination. It is desirable to establish that these vaccines are efficacious in the target animals in the field. The objective of this study was to carry out an independent evaluation of the immunogenicity of commercial EI vaccines in Thoroughbred horses on different premises and to provide data to veterinary surgeons who need evidence of vaccine efficacy to advise their clients about the relative benefits of different vaccines.

The immunogenicity of whole inactivated, subunit and canarypox recombinant EI vaccines can be evaluated by monitoring serological responses to the haemagglutinin (HA) antigen (Mumford et al., 1994a; Mumford et al., 1994b; Heldens et al., 2009; Toulemonde et al., 2005). HA is the major glycoprotein of influenza virus and is involved in attachment and entry of virus into the cell. Serum antibodies against HA
neutralise virus infectivity and correlate with protection (Mumford and Wood, 1992; Mumford, 2001). These antibodies can be detected by haemagglutinin inhibition (HI) or by single radial haemolysis (SRH) tests but the latter has been demonstrated to be more reproducible between laboratories (Mumford, 2000; Daly et al., 2007). The correlation between SRH antibodies and protection has been so well established that challenge studies to demonstrate efficacy are not required by the European Agency for the Evaluation of Medicinal Products (EMEA) for the substitution or addition of a new strain to an EI vaccine (EMEA, 1998). Experimental challenge studies and observations in the field suggest that horses with SRH antibody levels of 85mm$^2$ or greater are clinically protected against antigenically similar viruses and that those with antibody levels of 150mm$^2$ or greater are virologically protected and do not shed virus after challenge (Mumford et al., 1994b; Mumford, 2001; Newton et al., 2000a). Higher antibody levels may be required if the horses are vaccinated with vaccines that have not been updated with epidemiologically relevant strains (Daly et al., 2003).

In a previous study we compared the antibody responses of National Hunt horses in training to booster vaccination with one of the six EI vaccines available in Ireland (Gildea et al., 2011). The three whole inactivated (Prevac T Pro, Intervet; Duvaxyn IET Plus, Fort Dodge; Equilis Resequin, Intervet), two subunit (Equilis Equenza T, Intervet; Equip FT, Schering Plough) and a canarypox recombinant vaccine (ProteqFlu Te, Merial) available at the time of the study were compared. There was no significant difference between antibody responses induced following booster vaccination with any of the six vaccines. In order to eliminate the confounding effect of previous exposure to EI by natural infection or vaccination, the present study examined the serological responses of immunologically naive Thoroughbred weanlings, following primary vaccination with the five EI vaccines commercially available in Ireland at the time of this study. Two Intervet vaccines, Prevac T Pro and Equilis Equenza used in the study carried out in National Hunt horses were replaced with a subunit vaccine Equilis Prequenza Te.

Vaccine failure is most commonly reported in young racehorses (Newton et al., 2000a). When horses enter the training yards it is not uncommon for the same animals to receive different influenza vaccines over time. It is crucial that the vaccine used for the primary course of three doses stimulates a robust antibody response. The SRH antibody
responses to each of the five vaccines were monitored following the first three doses of vaccine, which were administered in accordance with the rules of the Turf Club. The aim of this study was to determine which of the vaccines elicited the highest antibody response in a randomised study in field conditions. High antibody levels correlate with protection against clinical disease and virus shedding therefore the use of a vaccine that elicits a strong humoral response will assist in the control of EI.

6.3 Materials and Methods

6.3.1 Horses
This study was carried out on a population of 66 unvaccinated Thoroughbred weanlings on four different stud farms in Ireland. The number of weanlings was 10, 11, 26 and 19 on premises one to four respectively. The population size on each premises was dictated by reliance on the cooperation of the stud owner to supply seronegative weanlings and agree to the necessary blood sampling regime. Weanlings of both genders were included in this study and they ranged in age from 159 to 297 days with a mean age of 235 days ± 3.97 SEM at the time of administering the first dose of vaccine (V1). The weanlings were all born in 2007 to mares that varied in age from four to 20 years of age at the time of parturition.

6.3.2 Vaccines
All vaccines were purchased commercially. The composition and type of adjuvant of each of the five vaccines included in this study are shown in Table 6.1. The multivalent vaccine Equilis Resequin (Intervet) combines EI and equine herpesvirus type 1 (EHV-1) and type 4 (EHV-4). In addition, Equilis Resequin was the only vaccine included in this study where a combined influenza/tetanus vaccine was not available. In the horses vaccinated with this product, tetanus toxoid (Intervet) was administered separately by the veterinary surgeon on the same days as Equilis Resequin.
<table>
<thead>
<tr>
<th>Vaccine/Manufacturer</th>
<th>Nature</th>
<th>Adjuvant</th>
<th>Strain/Antigenic Mass</th>
<th>Subtype</th>
<th>Lin</th>
<th>Tet Tox</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duvaxyn IE-T Plus (Fort Dodge)</td>
<td>Inact whole</td>
<td>Carbomer, Alum. Hydr.</td>
<td>A/eq/Prague/56 (15-18 µg) A/eq/Suffolk/89 (15-18 µg) A/eq/Newmarket/1/93(15-18 µg)</td>
<td>H7N7</td>
<td>H3N8</td>
<td>E</td>
<td>18 Lf</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H3N8</td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>Equilis Resequin* (Intervet)</td>
<td>Inact whole</td>
<td>Alum. Hydr., Immunostim</td>
<td>A/eq/Prague/56 (50 µg) A/eq/Newmarket/2/93 (20µg) A/eq/Newmarket/1/93 (20µg)</td>
<td>H7N7</td>
<td>H3N8</td>
<td>E</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H3N8</td>
<td>A</td>
<td>13</td>
</tr>
<tr>
<td>Equip FT (Schering Plough)</td>
<td>Subunit</td>
<td>ISCOM, Quillaic Acid derivative, Alum. phosphates</td>
<td>A/eq/Newmarket/77(10µg) A/eq/Kentucky/98 (1.4 µg) A/eq/Borlange/91(20µg)</td>
<td>H7N7</td>
<td>H3N8</td>
<td>A</td>
<td>100Lf</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H3N8</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>Equilis Prequenza Te (Intervet)</td>
<td>Subunit</td>
<td>ISCOM, chol., P. saponin, Phos. choline.</td>
<td>A/eq/Prague/56 (100 AU) A/eq/Newmarket/1/93 (50 AU) A/eq/Newmarket/2/93 (50 AU)</td>
<td>H7N7</td>
<td>H3N8</td>
<td>A</td>
<td>40 Lf</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H3N8</td>
<td>E</td>
<td></td>
</tr>
<tr>
<td>ProteqFlu-Te (Merial)</td>
<td>CPRV</td>
<td>Carbomer</td>
<td>A/eq/Kentucky/94 (≥ 5.2 log 10 FAID 50) A/eq/Newmarket/2/93 (≥ 5.2 log 10 FAID 50)</td>
<td>H3N8</td>
<td>A</td>
<td>&gt;30 IU</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>H3N8</td>
<td>E</td>
<td></td>
</tr>
</tbody>
</table>

Table 6.1: Vaccine product details

Lin = EIV H3N8 lineage; Tet Tox = Tetanus toxoid, No. = Number of horses; Inact whole = Inactivated whole, Alum. Hydr = Aluminium Hydroxide, E = European lineage; A = American lineage; Lf = Limes flocculation units, * = multivalent vaccine, N/A = not applicable, ISCOM = immune stimulating complex; chol = cholesterol, P. saponin = Purified saponin, Phos. choline= phosphatidylcholine, AU=antigenic unit, CPRV = Canarypox recombinant vaccine, FAID 50 = Fluorescent assay infectious dose; IU = International Unit.
6.3.3 Vaccination

The horses in this study were randomly allocated one of the five vaccines using the random number generator available within Microsoft Excel. The number of weanlings allocated each of the vaccine products is indicated in Table 6.1. Gender was not explicitly taken into account during randomisation of vaccine allocation. Vaccines were administered by deep intra muscular injection. The weanlings received their first two doses of their primary course (V1 and V2) five weeks apart followed by their third dose 26 weeks later (V3).

6.3.4 Collection of samples

Whole blood samples were collected from horses at nine different intervals as shown in Table 6.2. Samples were submitted to the laboratory following collection and serum was stored at -20°C until tested.

Table 6.2
Timetable for vaccination and collection of samples from horses

<table>
<thead>
<tr>
<th>Weeks</th>
<th>0</th>
<th>2</th>
<th>5</th>
<th>7</th>
<th>18</th>
<th>31</th>
<th>33</th>
<th>44</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccination</td>
<td>V1</td>
<td>V2</td>
<td>V3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling</td>
<td>S1</td>
<td>S2</td>
<td>S3</td>
<td>S4</td>
<td>S5</td>
<td>S6</td>
<td>S7</td>
<td>S8</td>
<td>S9</td>
</tr>
</tbody>
</table>

6.3.5 Serology

Antibodies against A/eq/Newmarket/2/93 (H3N8) a representative of the Eurasian lineage, A/eq/Kildare/92 (H3N8) a representative of the American lineage, A/eq/South Africa/4/03 (H3N8) a representative of the Florida sub-lineage of the American lineage and A/eq/Prague/56, the prototype H7N7 virus, were measured using the SRH test according to standard procedures and as previously described (Gildea et al., 2011). Control antisera against A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/2/93 (H3N8), A/eq/Newmarket/1/93 (H3N8) and A/eq/South Africa/4/03 (H3N8) from the European Directorate for the Quality of Medicines and Healthcare (EDQM), were included on
each plate as appropriate. The haemolytic zones resulting from the lysis of the antigen-coated sheep red blood cells by the antibody in the test sera were measured with a viewer and digital recording apparatus (Mitutoya, Aurora, Illinois, USA). The area of haemolysis was calculated and results were expressed in mm².

Mean H3N8 antibody values were calculated from SRH results obtained against the H3N8 antigens A/eq/Kildare/92, A/eq/Newmarket/2/93 and A/eq/South Africa/4/03 and a significant rise in antibodies was defined as an increase in the SRH level of 25mm² or 50% whichever was smaller between the successive serum samples (Newton et al., 2000a). For the purpose of this study, maternally-derived antibodies (MDA) were defined as an SRH antibody level of >25mm² for two or more H3N8 antigens at the time of S1. The antibody responses of five weanlings with MDA at the time of V1 were analysed separately to the 61 weanlings without MDA. The laboratory investigator was blinded to vaccine allocation to individual horses. The responses to tetanus toxoid and equine herpes virus (Equilis Resequin only) following vaccination were not examined during this study.

### 6.3.6 Statistical analysis

All statistical analysis was carried out using the open source package R version 2.8.1 (The R Foundation for Statistical Computing, Austria). Tests of significance were carried out at the $\alpha = 5\%$ level. Repeated measures analysis of variance with post hoc testing was carried out using Tukey’s Honest Significant Difference (HSD) (Hsu, 1996). The area under the curve (AUC) as described by Heldens et al., (2002) was calculated by the trapezoidal rule and used as the metric for the repeated measures analysis of antibody levels. This method takes into account the timescale over which protective antibody levels are maintained (Heldens et al., 2002). Performance of the vaccines at different time points was also compared using Tukey’s HSD (Hsu, 1996). A Chi squared test was used to examine the association between different vaccines and the incident of failure to seroconvert post vaccination.
6.4 Results

6.4.1 Course of antibody response

The pattern of antibody response to the H3N8 antigens was similar for all vaccines (Figures 6.1-6.3). Horses responded poorly to the first dose of vaccine (V1) (mean SRH level of 45.93 mm² ± 7.05 SEM for H3N8 antigens) but mounted a better immune response to the second dose administered five weeks later (V2). The mean SRH antibody levels for H3N8 antigens two weeks post V2 was 159.15 mm² ± 8.39 SEM which is consistent with virological protection. However, by thirteen weeks post V2 the mean SRH antibody levels for H3N8 had declined to 60.94 mm² ± 7.48 SEM i.e. a level below that associated with clinical protection. By 26 weeks post V2 i.e. the time of V3 these antibody levels had further declined and were similar to those at the time of V2. A strong antibody response was mounted to V3. Two weeks post V3 the mean SRH antibody levels for H3N8 antigens was 194.29 mm² ± 7.96 SEM but this level of antibody declined within the 13 weeks post V3 to 128.74 mm² ± 9.09 SEM. Thirteen weeks post V3 however; these SRH levels of 128.74 mm² ± 9.09 SEM were superior to those of 60.94 mm² ± 7.48 SEM thirteen weeks post V2. At 26 weeks post V3 samples were only received from 18 of the 61 horses due to the dispersal of horses into pre-training yards. These horses had a mean H3N8 SRH antibody level of 92.72 mm² ± 8.26 SEM. The H7N7 antibody response to the four vaccines which contained a virus of this subtype (Table 6.1) was analysed as a comparator to the H3N8 antibody response. The pattern of antibody response to the H7N7 antigen was similar to the response to the H3N8 antigens for all vaccines (data not shown).
Figure 6.1: Mean SRH antibody in vaccinated weanlings measured against A/eq/Kildare/92

Broken lines = SRH antibody level 85mm\(^2\) and 150mm\(^2\) correlating with clinical and virological protection respectively; error bars represent standard error of the mean.
Figure 6.2: Mean SRH antibody in vaccinated weanlings measured against A/eq/Newmarket/2/93

Broken lines = SRH antibody level 85mm$^2$ and 150mm$^2$ correlating with clinical and virological protection respectively; error bars represent standard error of the mean.
Figure 6.3: Mean SRH antibody in vaccinated weanlings measured against A/eq/South Africa/4/03

Broken lines = SRH antibody level 85mm$^2$ and 150mm$^2$ correlating with clinical and virological protection respectively; error bars represent standard error of the mean
6.4.2 Comparison of antibody response

The antibody responses of the horses vaccinated with Duvaxyn IET Plus against all three H3N8 antigens was significantly higher over all time points post vaccination than that of the horses vaccinated with the other products (Tukey’s HSD, p <0.0001; see Table 6.3). A Tukey’s HSD comparison between each of the five vaccine products was carried out at seven different time points post vaccination. Results of the significant differences between each of these time points are shown in Table 6.4. The final time point 26 weeks post V3 was excluded due to the loss of horses from the study at this time. The antibody response of the horses vaccinated with Duvaxyn IET Plus against each of the H3N8 antigens was significantly higher than that of the horses vaccinated with other product(s) at each time point.

### Table 6.3: Post hoc Tukey’s HSD comparisons

<table>
<thead>
<tr>
<th>A/eq/Kildare/92 (H3N8)</th>
<th>Mean difference for each pair (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-83.43, -22.97)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-68.98, -11.46)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-83.60, -25.57)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-89.51, -28.33)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-16.59, 42.55)</td>
<td>0.750</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-31.21, 28.44)</td>
<td>1.000</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-37.07, 25.64)</td>
<td>0.987</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-42.70, 13.97)</td>
<td>0.636</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-48.64, 11.25)</td>
<td>0.429</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Prequenza Te</td>
<td>(-34.52, 25.86)</td>
<td>0.995</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A/eq/Newmarket/2/93 (H3N8)</th>
<th>Mean difference for each pair (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-92.97, -34.05)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-79.83, -23.77)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Vaccine Pair</td>
<td>Mean Difference (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>------------------------------------------</td>
<td>--------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-86.30, -29.74)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-93.06, -33.43)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-17.11, 40.54)</td>
<td>0.800</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-23.57, 34.56)</td>
<td>0.986</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-30.30, 30.83)</td>
<td>1.000</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-33.84, 21.39)</td>
<td>0.972</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-40.63, 17.74)</td>
<td>0.820</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Prequenza Te</td>
<td>(-34.65, 24.20)</td>
<td>0.989</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vaccine Pair</th>
<th>Mean Difference (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/eq/South Africa/04/03 (H3N8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-87.32, -30.18)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-75.57, -21.21)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-84.23, -29.38)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-90.46, -32.63)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-17.60, 38.31)</td>
<td>0.849</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-26.25, 30.13)</td>
<td>1.000</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-32.44, 26.84)</td>
<td>0.999</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-35.20, 18.36)</td>
<td>0.911</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-41.46, 15.15)</td>
<td>0.708</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Prequenza Te</td>
<td>(-33.27, 23.80)</td>
<td>0.991</td>
</tr>
</tbody>
</table>

Strain in bold = SRH antigen; 95% CI = 95% Confidence interval for difference in mean zones of haemolysis generated between different vaccines; * Indicates statistically significant (P <0.05)
### Table 6.4: Tukey’s HSD of antibody response between vaccines at different sampling time points

<table>
<thead>
<tr>
<th>S2</th>
<th>S3</th>
<th>S4</th>
<th>S5</th>
<th>S6</th>
<th>S7</th>
<th>S8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kil 1&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Kil 1,2,3,4</td>
<td>Kil 1,4,5</td>
<td>Kil 1,2,3,4</td>
<td>Kil 1,3,4</td>
<td>Kil 1,2,3,4</td>
<td>Kil 1,2,3,4</td>
</tr>
<tr>
<td>NM 1,2,3,4</td>
<td>NM 1,2,3,4</td>
<td>NM 1,4</td>
<td>NM 1,2,3,4</td>
<td>NM 1,2,3,4</td>
<td>NM 1,2,3,4</td>
<td>NM 1,2,3,4</td>
</tr>
<tr>
<td>SA 1,2,3,4</td>
<td>SA 1,2,3,4</td>
<td>SA 1,3</td>
<td>SA 1,2,3,4</td>
<td>SA 1,2,3,4</td>
<td>SA 1,2,3,4</td>
<td>SA 1,2,3,4</td>
</tr>
</tbody>
</table>

S2 = two weeks post V1; S3 = at the time of V2; S4 = two weeks post V2; S5 = 13 weeks post V2; S6 = at the time of V3; S7 = two weeks post V3; S8 = 13 weeks post V3. Kil = A/eq/Kildare/92 (H3N8); NM = A/eq/Newmarket/2/93 (H3N8); SA = A/eq/South Africa/04/03 (H3N8); 1 = Significant difference between Duvaxyn IET Plus and Equilis Resequin; 2 = Significant difference between Duvaxyn IET Plus and Equip FT; 3 = Significant difference between Duvaxyn IET Plus and Equilis Prequenza Te; 4 = Significant difference between Duvaxyn IET Plus and ProteqFlu Te; 5 = Significant difference between Equip FT and ProteqFlu Te

#### 6.4.3 Area under curve (AUC)

The AUC of the SRH levels of the horses vaccinated with Duvaxyn IET Plus against all three H3N8 antigens was significantly higher than that of the horses vaccinated with the other products (Tukey’s HSD, p < 0.05; see Table 6.5).

### Table 6.5: Tukey’s HSD multiple comparisons of mean AUC

<table>
<thead>
<tr>
<th>A/eq/Kildare/92 (H3N8)</th>
<th>Mean difference for each pair (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-1677.31, -102.23)</td>
<td>0.018*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-1933.55, -385.18)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-1955.82, -419.67)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-2432.40, -601.27)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-1047.31, 508.13)</td>
<td>0.876</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-1069.61, 473.67)</td>
<td>0.826</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-1545.62, 291.50)</td>
<td>0.333</td>
</tr>
<tr>
<td>Comparison</td>
<td>Mean difference for each pair (95% CI)</td>
<td>P value</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>----------------------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-786.39, 729.62)</td>
<td>1.000</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-1264.61, 549.67)</td>
<td>0.815</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Prequenza Te</td>
<td>(-1231.02, 572.84)</td>
<td>0.854</td>
</tr>
<tr>
<td><strong>A/eq/Newmarket/2/93 (H3N8)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-1771.15, -194.89)</td>
<td>0.006*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-1895.98, -346.46)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-2084.41, -547.11)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-2352.11, -519.60)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-916.50, 640.10)</td>
<td>0.988</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-1104.96, 439.47)</td>
<td>0.761</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-1372.09, 466.41)</td>
<td>0.658</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-953.12, 564.03)</td>
<td>0.955</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-1222.45, 593.18)</td>
<td>0.876</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Prequenza Te</td>
<td>(-1022.70, 782.51)</td>
<td>0.996</td>
</tr>
<tr>
<td><strong>A/eq/South Africa/04/03 (H3N8)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Equilis Resequin – Duvaxyn IE-T Plus</td>
<td>(-1592.56, -117.53)</td>
<td>0.014*</td>
</tr>
<tr>
<td>Equip FT – Duvaxyn IE-T Plus</td>
<td>(-1818.98, -368.96)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Duvaxyn IE-T Plus</td>
<td>(-1976.71, -538.13)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>ProteqFlu Te - Duvaxyn IE-T Plus</td>
<td>(-2289.92, -575.09)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Equip FT – Equilis Resequin</td>
<td>(-967.25, 489.40)</td>
<td>0.896</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equilis Resequin</td>
<td>(-1125.01, 320.25)</td>
<td>0.544</td>
</tr>
<tr>
<td>ProteqFlu Te – Equilis Resequin</td>
<td>(-1437.68, 282.76)</td>
<td>0.351</td>
</tr>
<tr>
<td>Equilis Prequenza Te – Equip FT</td>
<td>(-873.31, 546.41)</td>
<td>0.970</td>
</tr>
<tr>
<td>ProteqFlu Te – Equip FT</td>
<td>(-1188.06, 510.98)</td>
<td>0.809</td>
</tr>
</tbody>
</table>
### 6.4.4 Maternally derived antibodies

Five weanlings in this study had SRH antibodies at the time of first vaccination (V1/S1) which, in the absence of a history of respiratory disease, were presumed to be maternal in origin. These animals were identified as weanling 1-5 for the purpose of analysis and at the time of V1 were 202, 208, 243, 226 and 171 days old respectively. Weanling 1 was vaccinated with Duvaxyn IE-T Plus. This animal failed to respond to V1 but did mount a response to V2 and V3. Weanlings 2 and 3 were vaccinated with Equilis Resequin, neither of these weanlings responded to V1 or V2 but they did respond to V3. Weanling 4 was vaccinated with Equilis Prequenza Te. This animal did not respond to V1 but did respond to V2 and V3. Finally, weanling 5 was vaccinated with ProteqFlu Te. This animal did not respond to V1 or V2 but did respond to V3. Figure 6.4 illustrates the antibody responses of each weanling with MDA to vaccination and these data are summarised in Table 6.6. At 26 weeks post V3 samples were only received from one of the five weanlings with MDA (weanling 5) due to the dispersal of horses into pre-training yards. Therefore this time point was not included in Figure 6.4. There was no statistical difference in overall vaccine performance as measured by AUC between horses with and without MDA at the time of V1 (P >0.05 for A/eq/Kildare/92 (H3N8), A/eq/Newmarket/2/93 (H3N8) and A/eq/South Africa/04/03 (H3N8).
Figure 6.4: Mean H3N8 SRH antibody levels in vaccinated weanlings with MDA at the time of V1

Weanlings 1 -5 vaccinated with Duvaxyn IET Plus (weanling 1), Equilis Resequin + T (weanlings 2 & 3), Equilis Prequenza Te (weanling 4) and ProteqFlu Te (weanling 5). Sampling time point S9 was excluded from this analysis as only one weanling was available at that time. Broken lines = SRH antibody level 85mm$^2$ and 150mm$^2$ correlating with clinical and virological protection respectively.

6.4.5 Failure or delay in response to vaccination

Failure to seroconvert to H3N8 antibodies was examined in each of the vaccine groups two weeks post vaccination (V1, V2, V3). The five weanlings with MDA at the time of first vaccination were excluded from this analysis. Classically, seroconversion is a change in the serological status of an animal from negative to positive. When the animals with MDA were excluded, 57 of the remaining 61 weanlings were seronegative and four had barely detectable SRH levels ($\leq$14mm$^2$) at the time of V1. In this study, a seroconversion was defined as an increase of $\geq$25mm$^2$ in antibody level. A poor
responder was defined as a horse that did not mount a mean H3N8 SRH antibody response of $\geq 25\text{mm}^2$ post vaccination. The number of poor responders to each vaccine following V1, V2 and V3 are summarised in Table 6.7. There was a significant difference in the incidence of failure to seroconvert between each of the five vaccine groups following V1 (P<0.001) and V2 (P<0.05). No animals failed to mount a significant antibody response following V3. A delayed response to vaccination was observed in one of the 11 horses (9%) vaccinated with Equilis Resequin. This horse failed to respond to V1 two weeks post vaccination however responded two weeks later i.e. four weeks post vaccination. The antibody response of these horses to the three doses of the different vaccines is summarised in Table 6.6.
<table>
<thead>
<tr>
<th>Vaccine</th>
<th>V1</th>
<th>S2</th>
<th>V2</th>
<th>S4</th>
<th>S5</th>
<th>V3</th>
<th>S7</th>
<th>S8</th>
<th>S9*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duvaxyn IET Plus</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (1)</td>
<td>39.82</td>
<td>36.83</td>
<td>28.23</td>
<td>142.22</td>
<td>59.24</td>
<td>14.14</td>
<td>240.29</td>
<td>187.87</td>
<td>N/A</td>
</tr>
<tr>
<td>Poor responders (0)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Normal (12)</td>
<td>1.17</td>
<td>94.67</td>
<td>81.51</td>
<td>206.36</td>
<td>111.85</td>
<td>63.26</td>
<td>247.07</td>
<td>192.80</td>
<td>150.92</td>
</tr>
<tr>
<td><strong>Equilis Resequin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (2)</td>
<td>53.94</td>
<td>37.26</td>
<td>32.84</td>
<td>32.83</td>
<td>0.00</td>
<td>0.00</td>
<td>128.35</td>
<td>67.05</td>
<td>N/A</td>
</tr>
<tr>
<td>Poor responders (6)</td>
<td>1.19</td>
<td>9.89</td>
<td>9.27</td>
<td>109.03</td>
<td>15.75</td>
<td>6.36</td>
<td>166.93</td>
<td>105.45</td>
<td>98.36</td>
</tr>
<tr>
<td>Normal (5)</td>
<td>0.00</td>
<td>50.85</td>
<td>56.14</td>
<td>153.55</td>
<td>56.91</td>
<td>36.10</td>
<td>188.58</td>
<td>128.74</td>
<td>74.07</td>
</tr>
<tr>
<td><strong>Equip FT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (0)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Poor responders (11)</td>
<td>0.52</td>
<td>6.15</td>
<td>5.03</td>
<td>167.38</td>
<td>54.74</td>
<td>28.04</td>
<td>197.97</td>
<td>129.55</td>
<td>76.82</td>
</tr>
<tr>
<td>Normal (3)</td>
<td>0.00</td>
<td>119.07</td>
<td>67.48</td>
<td>215.23</td>
<td>103.14</td>
<td>43.44</td>
<td>193.14</td>
<td>123.23</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Equilis Prequenza Te</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (1)</td>
<td>53.53</td>
<td>55.77</td>
<td>43.29</td>
<td>128.53</td>
<td>41.11</td>
<td>20.00</td>
<td>165.96</td>
<td>116.15</td>
<td>N/A</td>
</tr>
<tr>
<td>Poor responders (1)</td>
<td>0.00</td>
<td>6.07</td>
<td>5.44</td>
<td>123.80</td>
<td>6.09</td>
<td>5.67</td>
<td>89.45</td>
<td>16.64</td>
<td>6.52</td>
</tr>
<tr>
<td>Normal (12)</td>
<td>0.00</td>
<td>44.60</td>
<td>30.17</td>
<td>161.32</td>
<td>51.92</td>
<td>19.59</td>
<td>169.43</td>
<td>109.43</td>
<td>84.94</td>
</tr>
<tr>
<td><strong>ProteqFlu Te</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (1)</td>
<td>28.35</td>
<td>25.93</td>
<td>21.88</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>151.02</td>
<td>67.26</td>
<td>11.89</td>
</tr>
<tr>
<td>Poor responders (7)</td>
<td>0.96</td>
<td>6.12</td>
<td>3.03</td>
<td>88.29</td>
<td>25.76</td>
<td>17.30</td>
<td>184.23</td>
<td>97.76</td>
<td>95.07</td>
</tr>
<tr>
<td>Normal (4)</td>
<td>0.00</td>
<td>84.18</td>
<td>70.67</td>
<td>186.68</td>
<td>78.38</td>
<td>20.13</td>
<td>198.44</td>
<td>124.60</td>
<td>62.77</td>
</tr>
</tbody>
</table>

Table 6.6: Mean H3N8 SRH antibody levels for weanlings with MDA, poor responders and normal weanlings

S2 = 2 weeks post V1; S4 = 2 weeks post V2; S5 = 13 weeks post V2; S7 = 2 weeks post V3; S8 = 13 weeks post V3; S9 = 26 weeks post V3. *At the time of S9, n = 5 normal responders vaccinated with Duvaxyn IET Plus, n = 2 and 2 poor and normal responders vaccinated with Equilis Resequin respectively, n = 2 poor responders vaccinated with Equip FT, n = 1 and 3 poor responders and normal responders vaccinated with Equilis Prequenza Te respectively and n = 1, 2 and 1 weanlings with MDA, poor responders and normal responders vaccinated with ProteqFlu Te respectively.
Table 6.7: Number of poor responders to each vaccine

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>2 weeks post V1</th>
<th>2 weeks post V2</th>
<th>2 weeks post V3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duvaxyn IE-T Plus (n=12)</td>
<td>0/12</td>
<td>0/12</td>
<td>0/12</td>
</tr>
<tr>
<td>Equilis Prequenza Te (n=13)</td>
<td>1/13</td>
<td>0/13</td>
<td>0/13</td>
</tr>
<tr>
<td>Equilis Resequin (n=11)</td>
<td>7/11</td>
<td>1/11</td>
<td>0/11</td>
</tr>
<tr>
<td>ProteqFlu Te (n=11)</td>
<td>7/11</td>
<td>3/11</td>
<td>0/9</td>
</tr>
<tr>
<td>Equip FT (n=14)</td>
<td>11/14</td>
<td>0/14</td>
<td>1/14</td>
</tr>
</tbody>
</table>

### 6.4.6 Effect of premises, gender, age of foal at the time of V1 and age of mare on antibody responses

ANOVA tests were carried out to examine if premises, gender of foal, age at the time of V1 or age of mare at the time of parturition influenced vaccination response as measured by AUC. This analysis did not include the five weanlings, which had MDA at the time of V1. No significant association between any of these factors and vaccination response was recorded (P >0.05).

### 6.5 Discussion

This study has provided a critical comparison of the antigenicity of the five EI vaccines available in Ireland. On the basis of SRH antibody levels the whole virus vaccine Duvaxyn IET Plus was superior to the other products tested. These findings differed from those of Mumford et al., (1994c) who found that an ISCOM vaccine was superior to an inactivated whole virus vaccine, but were consistent with those of a similar earlier study by our group (Cullinane et al., 2001). Some previous studies have suggested that monovalent EI vaccines are more effective than multivalent EIV/EHV vaccines (Cullinane et al., 2001; Holmes et al., 2006) however, Heldens et al., (2002b) demonstrated that Equilis Resequin elicited a comparable serological response to vaccine formulations containing EI only. In this study the multivalent vaccine Equilis Resequin elicited a comparable response to Equip FT, Equilis Prequenza Te and ProteqFlu Te. No local or systemic reactions were observed after vaccination with any of the five vaccines.
Ideally vaccines should induce local and systemic, antibody and cellular immune responses. Cellular immune and mucosal antibody responses were not evaluated in this study but ponies vaccinated with Equip F have been shown to develop an increase in EIV specific IFN-γ PBL indicating effective CMI priming (Paillot et al., 2008). Similarly, naïve horses vaccinated with a recombinant canarypox vaccine experienced an increase in EIV specific IFN-γ and IL-2 mRNA expression (Paillot et al., 2007; Adams et al., 2011). The induction of cellular immune responses to EIV has yet to be demonstrated for the other vaccines included in this study and the inclusion of an assessment of such responses in future comparative studies would be beneficial.

Overall, vaccine performance is best evaluated by virus challenge; however this is not practical in Thoroughbred yearlings in the field. At present the measurement of antibodies against HA by SRH is accepted as an indication of protection against virus challenge and as a useful method of assessing vaccine efficacy in the field (EMEA, 1998; Newton et al., 2000b). However, given the variation in types of product on the market a more comprehensive comparison could be achieved if additional correlates of protection were identified and quantitative assays for these correlates were standardised.

In this study there was no evidence that location, gender, age of foal at the time of V1 or age of dam at the time of parturition influenced the vaccination response of the weanlings. In a previous study of the SRH antibody responses of Thoroughbred yearlings and two year old racehorses to booster vaccination, no sexual dimorphism was observed (Newton et al., 2000b). However, in an evaluation of the risk factors for influenza infection in vaccinated racehorses, Barquero et al., (2007) identified male animals as being at approximately twice the risk of infection during the outbreak in Newmarket in 2003. This apparent gender difference was unrelated to differences in SRH antibody levels.

Interference of MDA with the immune response to EI vaccines is well established (Van Oirschot et al., 1991; van Maanen et al., 1992; Cullinane et al., 2001). It has been reported that MDA against EI persist for three to six months (Van Oirschot et al., 1991; van Maanen et al., 1992; Cullinane et al., 2001). Using highly sensitive ELISA tests Wilson et al., (2001) detected MDA in foals until at least age six months and suggested that they may interfere with the response to vaccines administered up to age six months.
and beyond. In a survey of weanlings aged 6-10 months and of unknown vaccination status, Gildea et al., (2010) found that over 70% were seronegative for H3N8 as measured by SRH. In this study of unvaccinated weanlings (5-10 months of age) 92% were seronegative while 8% had detectable MDA. The weanlings with MDA ranged in age from 171 to 243 days. The dams of two of these weanlings were vaccinated with Duvaxyn IET Plus three and a half, and four months prior to parturition. The dam of a third weanling was vaccinated with Equilis Equenza six months prior to parturition. The vaccination history of the remaining two mares whose foals had MDA at the time of V1 was unavailable. The weanlings failed to respond to the first dose of Duvaxyn IET Plus (one weanling), Equilis Resequin (2 weanlings), Equilis Preqenza Te (1 weanling) and ProteqFlu Te (1 weanling). No weanling with MDA was vaccinated with Equip FT. The weanlings vaccinated with Equilis Resequin and ProteqFlu Te also failed to respond to V2.

Cullinan et al., (2001) reported that a subunit vaccine (Nobi Equenza - Intervet) failed to prime foals when administered at an average age of three months and moreover that it appeared to induce a tolerance as defined by serological testing, to subsequent vaccination with the same product or with an inactivated whole virus vaccine (Fluvac - Fort Dodge). These findings were supported by studies in Kentucky and field data from the United Kingdom (Holland et al., 1999; Newton et al., 2000b). Furthermore, Barquero et al., (2007) reported a significantly reduced risk of influenza infection associated with the administration of V1 at 6-18 months of age compared to commencing vaccination at less than 6 months of age. However, Minke et al., (2007) demonstrated that immunisation of foals between 10 and 20 weeks of age with the canarypox recombinant vaccine ProteqFlu did not induce a detectable antibody response against the background of MDA but effectively primed the foals as revaccination resulted in a rapid and strong antibody response. In the study reported here, a clear anamnestic antibody response to V2 or V3 was elicited by all four vaccines administered to foals with MDA. The findings were consistent with those of Minke et al., (2007) in relation to the canarypox recombinant vaccine but also demonstrated that a subunit and two whole inactivated vaccines also effectively primed foals vaccinated at 29-35 weeks of age in the presence of MDA. Further studies are necessary to determine if vaccination with these products at an earlier age primes foals or induces serological tolerance. There was no statistical difference in overall vaccine
performance between weanlings with and without MDA in this study however, as there were only five animals with MDA, this could be due to lack of statistical power and merits further investigation with a larger group. By 13 weeks post V3, the weanlings with MDA vaccinated with Duvaxyn IET Plus and Equilis Frequenza Te had SRH levels similar to the other weanlings vaccinated with these vaccines i.e. levels consistent with virological and clinical protection respectively. The weanlings with MDA vaccinated with ProteqFlu Te and Equilis Resequin had lower SRH levels 13 weeks post V3 than the average level for the other weanlings vaccinated with these vaccines i.e. their levels were not consistent with clinical protection.

Poor responses to vaccination are seen in a proportion of horses and are believed to be an important factor in the transmission of virus in vaccinated populations (Newton et al., 2000a). It was reported that in the 1989 epizootic of influenza in the UK, poor responders that had antibody levels below 50mm$^2$ were 15 times more likely to be the index case within a premises than those with antibody levels above 50mm$^2$ (Wood, 1991; Mumford, 1999). In this study, 43% of weanlings had failed to seroconvert by two weeks post V1. These poor responders were observed in four of the five vaccine groups. They were not observed in the horses vaccinated with Duvaxyn IET Plus and only one horse vaccinated with Equilis Prequenza Te responded poorly. Seventy nine per cent of horses vaccinated with Equip FT and 64% of horses vaccinated with Equilis Resequin and ProteqFlu Te mounted a poor response to V1. Post V2 the incidence of poor responders was reduced to four of 61 horses, three of which were vaccinated with ProteqFlu Te. All of the poor responder horses mounted a response to V3. Poor responders vaccinated with Equilis Resequin, Equip FT and ProteqFlu Te mounted responses to V3 that were similar in magnitude to those of the seropositive horses vaccinated with these vaccines i.e. 13 weeks post V3 their average SRH level was consistent with clinical protection. The single poor responder vaccinated with Equilis Frequenza Te mounted a lower and much less durable response to V3 than the average response of the seropositive horses vaccinated with this vaccine. Thirteen weeks post V3 the average SRH level for horses vaccinated with Equilis Frequenza Te was consistent with clinical protection but this poor responder had an SRH level consistent with index case potential.
The findings of this study carried out in Thoroughbred weanlings were consistent with the observation of Newton et al., (2000a) that despite certified vaccination, SRH testing revealed no evidence of EI antibodies in acute blood samples in some Thoroughbred horses. However, the high incidence of poor responders had not been reported in previous experimental studies with Equilis Resequin, Equip F and ProteqFlu (Heldens et al., 2004; Toulemonde et al., 2005; Paillot et al., 2008; Bryant et al., 2010). This study differed in that Equip FT and ProteqFlu Te were used and a tetanus vaccine was administered at the same time as Equilis Resequin but there is no evidence that the presence of tetanus toxoid compromises the response to EI vaccination (Mumford et al., 1994a). Reduced immunogenicity of subunit vaccines compared with whole virus vaccines in unprimed subjects has been reported previously (Mumford, 1999; Bernstein et al., 1982) but in this study the subunit vaccine Equilis Prequenza Te was one of the two most immunogenic vaccines in unprimed horses.

The pattern of humoral antibody response was similar for all vaccines and for all antigens tested. Sera from vaccinated horses are cross-reactive to different virus strains (Mumford, 1992) and there was no evidence that any antigen strain matching effect, for example the use of A/eq/Newmarket/2/93 included in three vaccines (Equilis Resequin and Equilis Prequenza Te and ProteqFlu Te) confounded the SRH results. A rapid decline of antibody titre was observed after V2 and prior to V3 for all vaccines. The poor durability of the immune response of unprimed horses and ponies to two doses of EI vaccine has been documented previously (van Oirschot et al., 1991; van Maanen et al., 1992; Mumford et al., 1994a; Mumford et al., 1994b; Cullinane et al., 2001; Heldens et al., 2001). Cullinane et al. (2001) demonstrated that the inclusion of an additional booster vaccination between V2 and V3 decreased this window of susceptibility and afforded greater protection as indicated by higher haemagglutinin inhibition (HI) antibody titres. A study carried out by Heldens et al., (2009) found that Fjord foals vaccinated with Equilis Prequenza, had HI antibody levels at the time of V3 which exceeded those required for clinical protection (HI of 1:64) (European Pharmacopoeia, 2001). After challenge, the foals were partially protected, as the amount of virus shed and the severity of the clinical signs were significantly less than that demonstrated by non-vaccinated foals. It was claimed that this vaccine closed the “immunity gap” between V2 and V3 and was able to induce a protective immunity during the entire vaccination course. In our study, SRH levels of the horses vaccinated...
with this product declined within 13 weeks post V2 in a manner similar to other vaccines. At 13 and 26 weeks post V2 the mean H3N8 SRH antibody levels of horses vaccinated with Equilis Prequenza Te were 48.40 mm² ± 4.00 SE and 18.52 mm² ± 2.49 respectively. These results were comparable with those from horses vaccinated with Equilis Resequin, Equip FT and ProteqFlu Te (mean H3N8 SRH antibody levels <66mm² and 32mm² at 13 and 26 weeks post V2 respectively). However, the immunity gap may not be equivalent for all products as vaccines may differ in their ability to prime foals and to stimulate a cell mediated immune response. The clinical and virological protection induced by different vaccines after V2 would be best evaluated by a comparative experimental challenge study. All five vaccines produced SRH antibody levels greater than 150mm² consistent with virological protection, following V3. Antibodies elicited by four of the five vaccines started to decline rapidly after V3. Only the horses vaccinated with Duvaxyn IET Plus had antibodies consistent with virological protection 13 weeks post V3. Unfortunately the dispersal of horses into training yards resulted in the loss of all but 18 of the 61 (30%) horses to the study 26 weeks post V3. At this time these 18 horses had a mean H3N8 antibody level of >90mm² consistent with clinical but not virological protection. Four of the 18 horses (22%) sampled at this time had SRH antibody levels consistent with virological protection while 6 (33%) had levels consistent with clinical protection. Three of the four horses with antibody levels consistent with virological protection were vaccinated with Duvaxyn IET Plus while one horse was vaccinated with ProteqFlu Te. Of the six horses that had antibody levels consistent with clinical protection, two were vaccinated with Equilis Resequin, two with Equilis Prequenza Te, one with Duvaxyn IET Plus and one with Equip FT. The remaining 8 horses (44%) sampled at this time had SRH levels of less than 85mm². These results suggest that the claim of one year duration of protective immunity following booster vaccination by some vaccine companies may require careful evaluation by those responsible for the care of young horses. Comparable findings have been documented by Newton et al., (2000a) who identified a particularly high-risk period approximately four months following V3 in Thoroughbred yearlings and suggested that a programme of three doses followed by annual booster vaccination dose not provide sufficient immunity to protect all young horses.

In summary, the study demonstrates that independent evaluation of influenza vaccine performance in the field is critical to add to the body of knowledge gained from
experimental challenge experiments carried out for regulatory or marketing purposes. This is the first study to indicate that the whole virus vaccine Duvaxyn IET Plus elicited a better SRH antibody response in Thoroughbred weanlings than the multivalent whole virus vaccine Equilis Resequin, the subunit vaccines Equip FT and Equilis Prequenza Te or the canarypox recombinant vaccine ProteqFlu Te. As SRH antibody levels stimulated by vaccination is a highly reliable predictor of immunity against influenza this is an important finding that should be considered in addition to other parameters, when choosing which of these products to administer to young horses. No poor responders to Duvaxyn IET Plus were identified but a far higher incidence of poor responders was observed in the groups vaccinated with Equip FT, ProteqFlu Te or Equilis Resequin than was previously recorded. The susceptibility of such poor responders to influenza and their possible role in virus spread need to be elucidated. In this study vaccination of a small number of weanlings with MDA effectively primed the animals but in some cases their response to the primary course was lower than that of seronegative weanlings. Vaccination in the presence of MDA with the vaccines included in this study also merits further investigation with larger numbers of foals.

Acknowledgements
This study would not have been possible without the cooperation of the veterinary surgeons and stud owners to whom the authors are extremely grateful. All of the experimental work was funded by the Department of Agriculture under the National Development Plan and carried out at the Irish Equine Centre. The results will be submitted as part of a PhD thesis by Sarah Gildea to the University of Limerick.
6.6 References


FEI (2011) 'Federation Equestre Internationale. HM King Hussein Building, Chemin des Delices 9, 1006 Lausanne, Switzerland. 
http://www.horsesport.org/veterinary/vaccinations-and-health-requirements


Gildea, S., Arkins, S., Walsh, C. and Cullinane, A. (2011) 'A comparison of antibody responses to commercial equine influenza vaccines following annual booster
vaccination of National Hunt horses - a randomised blind study', *Vaccine*, 29(22), 3917-22.


King, E. L., Macdonald, D (2004) 'Report of the Board of Inquiry appointed by the Board of the National Horseracing Authority to conduct enquiry into the causes of the equine influenza which started in the Western cape in early December 2003 and spread to the Eastern Cape and Gauteng', *Australian Equine Veterinarian*, 23, 139-142.


Paillot, R., Kydd, J. H., MacRae, S., Minke, J. M., Hannant, D. and Daly, J. M. (2007) 'New assays to measure equine influenza virus-specific Type 1 immunity in horses', *Vaccine*, 25(42), 7385-98.


Chapter 7

Discussion and Conclusion
7.1 Discussion

All equine influenza (EI) viruses identified in Ireland from June 2007 until January 2010 belonged to the Florida sublineage of the American lineage. Clade 2 viruses predominated up to 2009. The first clade 1 virus was identified in November 2009 and, thereafter, clade 1 viruses were responsible for all the subsequent outbreaks identified. The formulation of EI vaccines is reviewed on an annual basis by an expert surveillance panel (ESP), which includes representatives from OIE and WHO. In 2010 the ESP recommended that the vaccines should not include a H7N7 virus or a H3N8 virus of the Eurasian lineage but that they should contain both a clade 1 i.e. A/eq/Ohio/03 and clade 2 i.e. A/eq/Richmond/07 virus of the Florida sublineage (OIE, 2010). Phylogenetic analyses of EI viruses identified during this study support these recommendations and indicate that they are epidemiologically relevant. ProteqFlu is the only vaccine available in Ireland to have been updated in line with the ESP recommendations from 2004 i.e. to include a clade 1 A/eq/South Africa/4/2003-like virus while all vaccines need to be updated in line with the 2010 recommendation.

Significant financial investment is required to update vaccine strains and vaccine companies have traditionally been slow to respond to this requirement. More recently, vaccine companies have favoured carrying out cross protection studies to demonstrate vaccine efficacy against recent virus isolates (Daly et al., 2007; Paillot et al., 2010; Bryant et al., 2010). Such studies may be of questionable benefit however as previous challenge experiments, serological evidence during field outbreaks and mathematical modelling studies have demonstrated that vaccine mismatch reduces protection against infection and virus shedding and that higher antibody levels are required to protect against heterologous challenge strains (Daly et al., 2003; Newton et al., 1999; Park et al., 2004). It is well recognised that the use of vaccines which contain out of date virus strains facilitates disease spread through subclinically infected animals. There is epidemiological evidence to suggest that outbreaks of EI which occurred in South Africa (1986 and 2003), India (1987), Hong Kong (1992) and Australia (2007) all originated following the importation of subclinically infected vaccinated horses from other countries where EI was endemic (Guthrie et al., 1999; King and MacDonald, 2004; Uppal et al., 1989; Powell et al., 1995; Callinan, 2008). This method of virus spread has played a major role in the global distribution of EI. Therefore, the updating
of vaccine strains and vaccine efficacy is of importance to all countries irrespective of their disease status. The inclusion of the historic H7N7 antigen in EI vaccines proved useful in this study to distinguish antibody levels induced following H3N8 exposure from antibody levels induced following EI vaccination. Work carried out by Heldens et al., (2001, 2002) demonstrated that multiple antigens administered to the horse do not adversely affect the immune response. This has also been demonstrated in humans where it is believed that multiple vaccines utilize only approximately 0.1% of the immune system (Offit et al., 2002). Nonetheless, there is no justification for the inclusion of H7N7 in four of the five commercially available vaccines in Ireland.

Surveillance serves a number of purposes including assisting with information on vaccine strain selection, alerting epidemiologists to disease trends and acting as an early warning system for horse owners, trainers and veterinarians. All of these processes are fundamental to influenza control programmes based on vaccination and serve to reduce the economic impact associated with EI outbreaks. While the numbers of EI viruses identified worldwide are low in comparison with human, avian and swine influenza viruses, surveillance has provided vital information on the evolution of the H3N8 virus strain and the importance of inadequately vaccinated horses in the transmission of viruses globally. In countries where sporadic large epizootics have occurred, a certain amount of in-depth epidemiological analysis has been undertaken. This data however, has largely focused on how the virus was introduced into the naïve population and how the virus was subsequently spread (Guthrie et al., 1999; King and MacDonald, 2004; Callinan, 2008). In previous studies, some important drivers in the transmission of EI have previously been recognised and include demographics structure, movement of horses, age and housing (Baguelin et al., 2010; Morley et al., 2000) however, the identification and emphasise of further important drivers and deterrent measures involved in EI transmission should therefore minimise disease spread within an endemic country and subsequent spread to populations in other countries with naïve populations.

Regions where EI is endemic such as America and Europe cover a large geographical land mass and account for an estimated 20% of the equine population of the world. In Ireland vaccine compliance is good within certain sectors of the equine industry.
However, there are vulnerable subgroups where vaccination is poorly executed, thus creating potential pockets of disease, facilitating virus spread and allowing the disease status to be maintained. In this study, H3N8 antibody levels indicative of virological protection was identified in 23% of the Irish horse population. While 32% were considered to have antibody levels indicative of clinical protection, 45% of the Irish equine population had H3N8 antibody levels suggestive of complete susceptibility in the event of an EI outbreak. Weanlings, teaser stallions and non-Thoroughbred horses and ponies were identified as the most susceptible. Nevertheless, susceptible animals were identified among all the populations identified. Such susceptible animals can undermine the overall level of herd immunity and facilitate a build up of virus challenge. Other secondary factors which could play a major role in the dissemination of virus at a population level include the structure of the population within Irish premises and the mixing patterns involved between the different subpopulations. Since it is estimated that one infected horse is capable on average, of infecting 10 completely naïve in contact horses (Glass et al., 2002) it is important that susceptible populations in Ireland are identified and vaccinated as appropriate.

In Ireland, the Turf Club has introduced a mandatory vaccination programme for racehorses. Mandatory vaccination is also a requirement for yearlings and horses in training prior to entry to the major Thoroughbred sales and for horses wishing to participate in Federation Equestre Internationale (FEI) competitions. The overall aim of such vaccination policies is to ensure sufficient herd immunity to protect equestrian events rather than individual horses. It has been suggested that 70% of a given population of horses need to be fully vaccinated to prevent epidemics of influenza (Baker et al., 1986-rationale for the use of influenza vaccines). Since its introduction, mandatory vaccination has been effective in limiting EI outbreaks and has prevented the requirement for cancellation of any major equestrian event in Ireland as a result of this disease. However, it is questionable as to whether the preliminary programme of three vaccine doses following by annual vaccination provides sufficient immunity to protect young horses from EI or individual training yards from sporadic outbreaks and whether such a rigorous vaccination programme is necessary for older horses with many years’ vaccination history. In this study EI vaccine efficacy was examined in two different equine populations, mature National Hunt horses and immunologically naïve Thoroughbred weanlings. In both groups a significant decrease in antibody levels was
observed 6 months post vaccination. In the National Hunt yard, analysis of the mean SRH levels post booster vaccination suggested that this population was clinically protected; however, analysis of the individual responses suggested that there was potential for vaccine breakdown. Vaccine failure is most commonly reported in young racehorses (Newton et al., 2000a). In the weanling population, low antibody levels were observed post V2 for all vaccines and antibodies elicited by four of the five vaccines started to decline rapidly after V3. These findings agreed with previous reports which also demonstrated the poor durability of the immune response of unprimed horses to two doses of EI vaccine (van Oirschot et al., 1991; van Maanen et al., 1992; Mumford et al., 1994a; Mumford et al., 1994b; Cullinane et al., 2001; Heldens et al., 2001) and with findings by Newton et al., (2000b) who identified a particularly high-risk period approximately 4 months following V3 in Thoroughbred yearlings. This broad standard schedule of vaccination introduced for horses thirty years ago was largely based on experience from human vaccines and not necessarily evidence-based in the target species (Newton et al., 2005). As all disease control measures should have a strong scientific base and justification, the results obtained during this study would suggest that further studies investigating more rigorous vaccination policies among young racehorses and more strategic vaccination among mature racehorses with many years’ vaccination histories may be beneficial. A proposed vaccination regime which may be worth examining in naïve horses could include additional booster vaccinations between second and third vaccine dose and six months post third vaccine dose for the first year of vaccination followed by six monthly boosters in the second year of vaccination.

7.2 Conclusions

Viral Characterisation: Genetic analysis of EI viruses serves as a scientific basis for investigating the source of epizootics and outbreaks both nationally and internationally. Moreover, genetic characterization is a requirement for identification of circulating viral strains and subsequent vaccine design. The surveillance data presented support the Expert Surveillance Panel recommendations that vaccines should not include an H7N7 virus or an H3N8 virus of the Eurasian lineage but that they should contain both a clade 1 and clade 2 virus of the Florida sublineage (OIE, 2010) and indicate that they are epidemiologically relevant.
Reducing Disease Spread: Effective preventative measures identified to reduce disease spread during EI outbreaks include isolation and clinical monitoring of new arrivals and horses returning from equestrian events, maintenance of effective boundaries between equine premises, avoidance of stabling in shared air spaces, serological testing of new arrivals and vaccination as appropriate and vaccination of horses, especially young horses and teasers at six monthly intervals.

Control measures for EI outbreaks include prompt isolation of suspected cases, implementation of strict biosecurity measures to avoid transmission by fomites, personnel and contaminated vehicles, rapid confirmatory diagnosis by RT-PCR, and booster vaccination of cohorts following early diagnosis. Implementation of these preventative and control measures should reduce the economic losses associated with outbreaks of EI.

Identifying EI-Susceptible Animals: Weanlings, teasers and non-Thoroughbred horses were identified, on the basis of their SRH antibody levels, as the equine subpopulations most susceptible to influenza virus. These results suggest that it would be advisable that weanlings commence their vaccination programme prior to attendance at public sales. There is currently no mandatory EI vaccination for entry to weanling sales in Ireland. The results of this study also demonstrate that the teaser population on stud farms is inadequately vaccinated and could play a major role in EI virus transmission to susceptible broodmares during an outbreak. Teaser stallions should be vaccinated against EI in accordance with the manufacturers’ instructions and preferably, receive a booster vaccination prior to each breeding season. Currently vaccination against EI is not required for entry to many of the non-Thoroughbred sales, fairs and local shows. The introduction of mandatory vaccination for participation in such events would reduce the risk of amplification of virus in this population and dissemination to other populations.

Responses to Vaccination in Different Equine Populations:
Mature Horses: There was no significant difference between the levels of antibody responses induced following booster vaccination of mature horses with any of the six
commercially available vaccines. However, based on the results of this study, it may be advantageous to monitor SRH levels and to vaccinate strategically. The presumption that annual revaccination is the minimum necessary to protect all horses against EI needs to be systematically evaluated. It has been demonstrated that shorter intervals are required for optimum protection of young horses and it may be that longer vaccination intervals are sufficient for older horses with several years of vaccination history.

**Weanlings:** Weanlings were identified as a particularly EI susceptible population based on SRH haemagglutinin antibody levels. Since these low antibody levels coincide with the winter high-risk seasonal period of influenza spread identified during the course of this study, weanlings are particularly at risk of contracting and disseminating EI within an equine premises. While vaccination of weanlings is not currently mandatory, it is customary to vaccinate this group shortly after weaning. There was no statistical difference in overall vaccine performance between weanlings with and without maternally derived antibody (MDA) in this study. A clear anamnestic antibody response to second (V2) and third (V3) vaccine dose was elicited by all four vaccines (canarypox recombinant, subunit and two whole inactivated vaccines) administered to foals at 29 to 35 weeks of age in the presence of MDA, suggesting that earlier vaccination in the presence of MDA may be possible.

A comparison of antibody responses to five commercially available EI vaccines showed that antibody responses of weanlings vaccinated with the whole virus vaccine Duvaxyn IET Plus were significantly higher than those of the horses vaccinated with any of the other four products. While one vaccine (ProteqFlu Te) included in this study has since been updated in line with the ESP recommendations of 2004 i.e. to include a clade 1 virus and other products (Equip F, ProteqFlu Te) claim to induce cellular immune responses post vaccination (Paillot et al., 2007; 2008; Adams et al., 2011) the use of Duvaxyn IET Plus to vaccinate naïve horses prior to entry into training yards should reduce the risk of disease spread in this high risk population. Similarly, poor responders were not observed among any horses vaccinated with Duvaxyn IET Plus and results of a previous study indicated that the proportion of horses responding poorly to vaccination has an important impact on the risks of EI for the population Baguelin et al., (2010). At present, little is known about CMI response in horses following natural infection/vaccination and until such data is available the
measurement of antibodies against EI using the SRH test is the most appropriate assay available to determine vaccination response and susceptibility.

The claim of immunity between V2 and V3 and for one year following V3 by vaccine companies may require careful re-evaluation, based on the results of antibody levels obtained during this study. A high incidence (43%) of poor responders was observed following V1. This high incidence had not been reported in previous experimental studies. While this poor response did not appear to impact on secondary immune responses, it may have significance for disease resistance in this period and may have specific implications for vaccine design for this population.

### 7.3 Future Work

Equine influenza surveillance serves to reduce the economic impact of disease by maintaining awareness and highlighting the emergence and international spread of antigenic variants (Cullinane et al., 2010). Surveillance to date is largely carried out in the UK, Ireland and France and to a limited extent in North America (Bryant et al., 2009; 2011; Gildea et al., 2011). A large number of countries do not participate in an active surveillance programme and, moreover, in many countries surveillance is only carried out to a limited extent. This current global disparity in surveillance could result in potentially biased information about the relative prevalence of EI viruses and may facilitate novel viruses circulating undetected for several years. Previously, inadequate surveillance of the swine population may have assisted with the emergence of the swine-origin influenza pandemic in 2009. Genetic analysis indicated that this virus, derived from a triple recombinant virus (swine, human, avian), had been circulating in swine populations, undetected, for at least ten years (Smith et al., 2009).

RNA viruses are characterised by their ability to rapidly generate genetic variation. Genetic reassortment of gene segments of both human and animal influenza A viruses have resulted in new pandemic strains emerging. Thus, amplification and analysis of the entire influenza A virus genome by PCR of human, swine and avian isolates is not uncommon in an effort to identify any newly emerging strains. As yet, EI virus is not believed to have played a major role in genetic reassortment or the emergence of new
virus strains in humans. Traditionally, analysis of EI isolates is carried out by genetic characterisation of the surface glycoprotein HA1 gene or antigenic characterisation using mono-specific ferret antisera in the haemagglutinin inhibition test. Recently, phylogenetic and antigenic analysis of the neuraminidase (NA) and HA genes of H3N8 EI viruses isolated in Greece from 2003-2007 has indicated that reassortment of EI virus has occurred between genetically distinct co-circulating strains (Bountouri et al., 2011). Similarly in a study carried out by Bryant et al., (2009) the virus A/eq/Lincolnshire/1/06 was characterised as a member of the Florida sublineage clade 2 by HA gene sequence, however, analysis of the NS gene indicated that this virus belonged as a member of the Eurasian lineage. Such results suggest reassortment between viruses. Reassortment either within EI virus lineages or between EI virus and influenza virus in other species would have implications not only for EI vaccine efficacy but for human and animal welfare globally. Therefore it is advisable that future surveillance should include routine whole genome sequencing of EI viruses.

At present, single radial haemolysis (SRH) HA antibody levels are considered to be the best correlate of protection and indication of immune status against EI. The current suite of commercially available vaccines have been designed predominantly with a view to induction of a humoral protective response, rather than sustained cell mediated immunity (CMI). This is in contrast to immunity stimulated by natural infection which is more robust and durable due to the stimulation of both humoral and cellular immune responses. Limited previous studies on inactivated EI vaccines have demonstrated that ponies vaccinated with Equip F developed an increase in EIV specific IFN-γ PBL indicating effective CMI priming (Paillot et al., 2008) and that naïve horses vaccinated with a recombinant canarypox vaccine experienced an increase in EIV specific IFN-γ and IL-2 mRNA expression (Paillot et al., 2007; Adams et al., 2011). However, there have been few studies to date to measure the levels of major histocompatibility complex (MHC) restricted influenza specific cytotoxic T cells, following either vaccination or infection. The demonstration that some animals with extremely low HA antibody levels are protected against EI when challenged suggests that CMI plays an important role in disease resistance (Hannant et al., 1988). Ideally, surveillance studies should take CMI status into account when documenting susceptibility within an endemic population. This is an important future objective. Similarly, knowledge of the pattern of CMI following vaccination, how SRH antibody levels reflect CMI and what
aspects of CMI correlate with protection may have long-term implications for the duration and level of protection induced by currently available vaccines and for future vaccine design. Future vaccine studies could include the collection of paxgene (Pre AnalytiX) blood RNA samples which allow for the immediate preservation of RNA profile. The examination of IL-1β and IL-6, two pro-inflammatory cytokines (Quinlivan et al., 2007) IL-4 and IL-10, cytokines involved in B cell differentiation (Simpson et al., 1997) and IFN-γ a marker of cellular mediated immunity by quantitative RT-PCR of mRNA may prove informative in achieving a better understanding of cellular immune response in the horse post vaccination.

The method of vaccine presentation is a significant determinant of the nature of the immune response. Paillot et al., (2006) and others (Mazanec et al., 1995; Tamura et al., 1991) have described the importance of IgA and the mucosal immune response in preventing infection and reducing viral shedding post-infection. Hannant et al., (1999) demonstrated the ability of an intranasal vaccination to induce a local immune response, involving virus specific neutralisation antibodies and IgA antibodies. More recently, a cold adapted temperature sensitive modified live intranasal equine influenza vaccine has become commercially available in North America and has demonstrated clinical protection in naïve animals for up to six months after a single vaccine dose, sometimes in the absence of significant serum antibody levels (Townsend et al., 2001). Thus vaccination with this product induces other immune mechanisms, probably including respiratory mucosal immunity. The administration of DNA vaccines in many species including horses has also demonstrated stimulation of immune responses other than humoral immunity (Soboll et al., 2003; Lunn et al., 1999). Current systemic vaccine strategies available in Ireland do not maximise mucosal and or cellular immune responses. Alternative vaccine presentation strategies may serve to elicit a more sustained or protective immune response and evaluation of such strategies may prove beneficial in reducing virus spread in an endemic country when compared to the traditional inactivated vaccination approach.

Adjuvants are generally included in systemic vaccines to enhance the level and durability of the antibody response. In the past few decades the introduction of new adjuvants has subsequently resulted in an increase in the effectiveness of conventional vaccines. In this study significant differences were observed in antibody levels induced
following vaccination with commercially available vaccines with similar antigenic content however with different adjuvant delivery systems. This would suggest that different adjuvants may contribute greatly to vaccine efficacy and that further studies regarding this aspect of vaccine design may prove instrumental in the control of EI.

7.4 References


King, E. L., Macdonald, D (2004) 'Report of the Board of Inquiry appointed by the Board of the National Horseracing Authority to conduct enquiry into the causes of the equine influenza which started in the Western cape in early December 2003 and spread to the Eastern Cape and Gauteng', Australian Equine Veterinarian, 23, 139-142.


vaccines overwhelm or weaken the infant's immune system?’, *Pediatrics*, 109(1), 124-9.


Paillot, R., Kydd, J. H., MacRae, S., Minke, J. M., Hannant, D. and Daly, J. M. (2007) 'New assays to measure equine influenza virus-specific Type 1 immunity in horses', *Vaccine*, 25(42), 7385-98.


and antiviral cytokine expression in vaccinated and unvaccinated horses exposed to equine influenza virus', *Vaccine*, 25(41), 7056-64.


