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Study of vaccinations used for the 2019 Equine Influenza outbreak in the UK

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1. List of Abbreviations

equine influenza virus - **(EIV)**
Florida sub lineage clade 1- (**FCL1)**
British Horseracing Authority - **(BHA**)
International Federation for Horseracing Authorities - **(IFHA)**
International Federation for Equestrian Sports - **(FEI)**
primary vaccine - **(V1)**
secondary vaccine - **(V2)**
booster vaccine - **(V3)**
Florida clade 2 - **(FCL2)**
influenza A virus - **(IAV)**
influenza B virus - **(IBV)**
influenza C virus – (**ICV)**
influenza D virus - **(IDV)**
RNA-dependent RNA-polymerase complex - **(RdRp)**
ribonucleoprotein complex - **(RNP)**
nucleoprotein - **(NP)**
haemagglutinin - **(HA)**
neuraminidase - **(NA)**
matrix protein-1 - **(M1)**
matrix protein-2 – **(M2)**
non-structural proteins - **(NS1)**
nuclear export proteins - **(NEP)**
red blood cells - **(RBCs)**
heamagglutinin-esterase-fusion - **(HEF)**
sialic acid - **(SA)**
messenger RNAs - **(mRNAs)**
complimentary copy - **(cRNA)**
complementary RNPs - **(cRNPs)**
heamaggluttination inhibition - **(HI)**
single radial haemolysis - **(SRH)**
direct fluorescent assay – **(DFA)**
European Directorate for the quality of medicines - **(EDQM)**
differentiation vaccinated animals to infected animals - **(DIVA)**
European Horserace Scientific Liaison Committee - **(EHSLC)**
British Equine Veterinary Association - **(BEVA)**
Consensus- based approaches and design of computationally-optimized broadly reactive antigens - **(COBRA)**
standard of care - **(SOC)**

2. Abstract

In 2019 there was an equine influenza epidemic in the UK and Ireland with 228 laboratory confirmed outbreaks throughout the UK and Ireland, ranging mainly from January through to August. The causative agent of this particular outbreak was the Equine influenza virus (EIV), Florida sub lineage clade 1 (FCL1) of H3N8, from the *Orthomyxoviridae* family, which is predominantly seen in North America.

As a result of this outbreak, the British Horseracing Authority (BHA), who are a member of the International Federation for Horseracing Authorities (IFHA), cancelled racing events throughout the UK from the 7th to the 13th of February; after this opening the events with strict biosecurity and only allowing competitors from laboratory confirmed influenza free yards.

Vaccination against EIV first became obligatory in the 1980s in UK, Ireland and France in the racing industry after a major Equine influenza outbreak in 1979, which lead to the cancellation of several race meetings. Vaccination is now used prophylactically in these countries as well as many others, such as the USA and Japan.

There are many protocols in place for timing the administration of the vaccination. Vaccination should be administered at 6 months with two preceding vaccination followed by a yearly or 6-monthly booster vaccine depending on the country.

Currently there are three types of vaccination available for use against equine influenza, a recombinant canarypox-based vaccine, an inactivated vaccine, and an immune stimulating complex-based subunit vaccine. subunit vaccine.

The current legislation in Ireland for vaccination is under two main bodies, The Irish Horse Racing body and the International Federation for Equestrian Sports (FEI). In the UK the current legislation is under the British Horseracing Association and the FEI. The protocol is similar in both countries with some deviations in the timing between the vaccinations used, therefore it is recommended to follow the guidelines for each individual product.

The objective of this literature review is to look at the vaccinations used, the vaccination protocols in place at the time of the outbreak and the effect that antigenic drift had on the number of horses effected in past outbreaks as well as the outbreak in 2019.

3. Introduction

Equine influenza virus (EIV) is from the *Orthomyxoviridae* family, and is composed of 8 segments of single-stranded negative sense RNA encoding 10 genes (Bryant , et al., 2009). It is present worldwide with the exception of New Zealand and Iceland; and Australia until 2007.

Equine influenza is a highly contagious disease, that spreads rapidly with a high morbidity and low mortality. The disease is associated with high financial loss through veterinary bills and revenue loss through cancellation of racing or other equestrian events. Proof of this has been seen with different outbreaks throughout the years such as, in South Africa in 1986 to a more recent outbreak in Australia in 2007 costing the government over 263 million Australian dollars in assistance to the equine industry (Paillot & El- Hage, 2016).

Horses aged 1 to 5 are most susceptible to infection. The strong interferon response to the virus infection is mainly responsible for the general clinical signs. The main symptoms are anorexia, fever and respiratory symptoms. Clinical signs depend on the immune system of each individual animal, ranging from mild respiratory symptoms to severe disease in immune-compromised animals.

The resistance of the virus is relatively good compared to other enveloped viruses; it can survive in the environment for several hours up to a few days. A.J Guthrie's paper on the outbreak in South Africa in 1986 proved that part of the spread of the virus was due to environmental contamination as there was no direct contact between a proportion of the infected horses, only that they were transported on the same vehicle that was not disinfected in between shipments (Guthrie, et al., 1999). Nevertheless, the virus will be inactivated by drying out, high temperature, UV light, and most detergents as the virus is enveloped.

Previously the A/equi/Prague/56/H7N7 strain of the equine influenza virus was the most predominate cause of equine influenza, which was first isolated in eastern Europe in 1956. This strain has not been identified since 1977 (Bryant , et al., 2009).

It has now been replaced by H3N8 which was first discovered in 1963, when it caused a major epidemic in the USA. This subtype was initially thought to be a single lineage but further studies showed that two lineages emerged from the early 1980s. The lineages were primarily named geographically as American and Eurasian lineage but after isolating the virus, it was soon discovered that the American lineage was circulating in Europe.

The American lineage was then further sub-divided into the South-American sublineage, the Kentucky sublineage and the Florida sublineage. Further testing by the HA gene sequence showed a divergence in the Florida sublineage to Clade 1, which includes strains similar to the A/eq/Wisconsin/03–virus and Clade 2 which includes strains similar to the A/eq/Newmarket/5/03–virus (Bryant , et al., 2009). Clade 1 viruses are predominating in North and South America, but have not been identified in the UK since 2009 and in Ireland since 2010.

The obligatory vaccination protocol first came into place in the racing industry in UK, Ireland and France in the early 1980s after a major equine influenza outbreak in 1979 which lead to the cancellation of several race meetings. The vaccination programme included a primary vaccine (V1) and a secondary vaccine (V2) given no less than 21 days and no more than 92 days apart. A booster vaccine (V3) was then given no less than 150 and no more than 215 days after the second vaccine. Following the initial immunization program, an annual booster vaccination was required (Cullinane & Gildea, 2013).

The 2019 UK and Ireland outbreak was caused by a Florida sub lineage Clade 1 (FCL1) A/equi/2/Miami/H3N8 strain. Florida clade 2 (FCL2) was the predominant cause of equine influenza in the UK since 2009. Therefore, many horses were naïve and lacked immunity to this particular strain leading to rapid spread in yards and events, also leading to vaccinated horses becoming infected. In naïve horses the incubation period can be as short as 24 hours but in vaccinated horses it may be three to five days. Equine influenza is a highly contagious disease, and once it was first detected in January 2019 the numbers rapidly increased with outbreaks spiking in February and then again in June 2019.

4. Causative agent

The influenza virus belongs to the family *Orthomyxoviridae*, with four main genera (each including one species) *Alphainfluenzavirus* (species: influenza A virus; IAV), *Betainfluenzavirus* (species: influenza B virus; IBV), *Gammainfluenzavirus* (species: influenza C virus; ICV) and Deltainfluenzavirus (species: influenza D virus; IDV), which can all infect mammals (Hutchinson, 2018).

The virus has a segmented negative sense RNA genome, IAV and IBV have eight segments, while ICV and IDV have seven segments.

Clinically, the most important is influenza A virus, which is responsible for most epidemics in both in humans and animals.

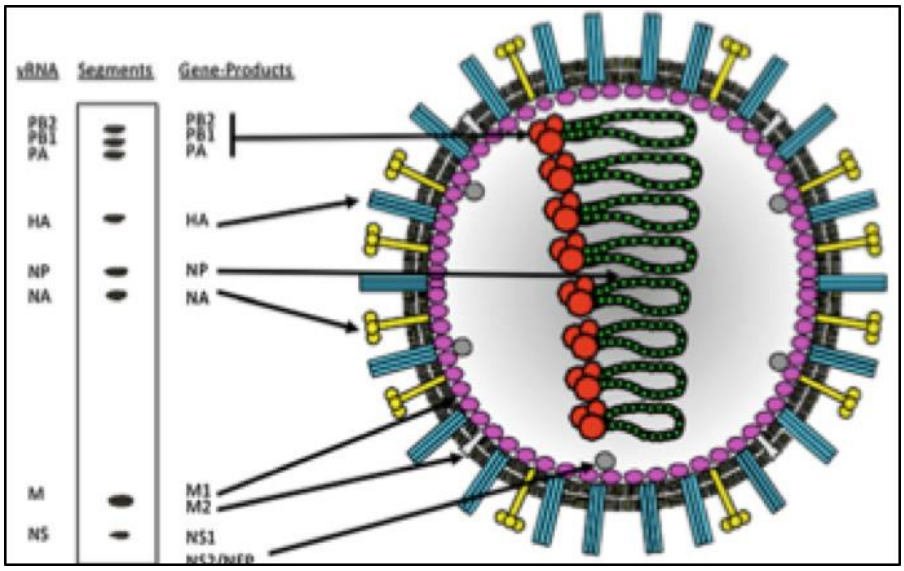

Figure 1. The structure of influenza A particle (Pleschka, 2012)

Structurally the virus has a lipid envelope formed from host cell and 9 or 10 structural viral proteins. **Figure 1** shows the 8 segments of the viral RNA. Segments 1 to 3 are encoding the components of the RNA-dependent RNA-polymerase complex (RdRp), PB2, PB1, and PA, all of which are linked to the ribonucleoprotein complex (RNP). The nucleoprotein (NP) that is the main component of the RNPs is encoded by segment 5 and produces genus specific antigens.

Segments 4 and 6 encode the viral surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). Matrix protein-1 (M1) is translated from mRNA of segment 7 and makes up the inner layer of the virion. Matrix protein-2 (M2), which is also encoded by

segment 7 functions as a pH-dependent ion channel for decapsidation. The last segment contains the sequence for non-structural proteins (NS1) and the nuclear export proteins (NEP). The latter is associated with Matrix 1 (Pleschka, 2012).

An essential virulence factor of influenza A viruses is the non-structural protein (NS)-1. Based on its coding sequence, two distinct groups (allele A and B) are established. Allele A genes are found in horses, humans, swine and birds, whereas allele B is mainly found in avian influenza viruses. NS1 functions as an antagonist of the innate immune response by blocking the activation of interferons via regulatory factor-3 (Bryant , et al., 2009).

The genome structure of the virus is of great importance as it allows for genetic mutation, recombination and new strains to be formed, this being one of the major problems in eradicating or even preventing outbreaks.

HA and NA are important factors for the antigenicity and together they determine the serotype. There are currently 18 known subtypes of HA and 11 subtypes of NA (Wu & Wilson, 2020). HA is the viral antireceptor protein that connects to the surface of red blood cells (RBCs) and attaches to the sialic acid receptors of the host cells. These receptors are mainly found on mucous membranes. HA undergoes a series of maturation steps that are dependent on interactions with the host cell. The final maturation step is the protease cleavage of the HA0 into its subunits HA1 and HA2 which is imperative for the function of HA and the infection of the cell. The cleavage activation is mediated by extracellular or cellular enzymes (Pleschka, 2012).

In some of the more virulent subtypes such as H5 and H7, there are several basic amino acid inserts at the cleavage site that are activated by ubiquitous endoproteases such as furin. As these proteases are present in almost every cell, avian viruses containing these multibasic cleavage sites can infect most host organs, leading to highly pathogenic avian influenza.

In all other HA subtypes there are monobasic cleavage sites with less amino acids that are only activated after the release by extracellular proteases. These include trypsin, which is present only in certain tissues, mainly the respiratory tract; trypase clara, which is secreted from the bronchiolar epithelial cells and inflammatory active proteases such as urokinase, kallikrein, and thrombin (Pleschka, 2012).

NA is responsible for budding of progeny virus particles from the host cell and thus for virus release and spread.

The replication of the virus is quite complex and is divided into three main steps, attachment and entry of the virus into the host cell, genome transcription and replication of the viral RNA, finally assembly and release of the progeny virus from the host cell.

The haemagglutinin proteins of IAV and IBV and the haemagglutinin-esterase-fusion (HEF) proteins of ICV bind with the sialic acid (SA) receptor of the host cell. The structure of the SA receptor differs depending on the species affected, birds have SA2-3GAL, humans have SA2-6Gal, while swine have both SA2-3GAL and SA2-6Gal. This means that the HA of viruses adapted to avian SA receptors will find it hard to bind to human SA receptors (Pleschka, 2012).

Following the binding of the virus HA and the host cell SA receptor endocytosis occurs. Once inside the cytoplasm of the host cell there is fusion of the viral envelope with lysosomes which release digestive enzymes and produce an acid medium. This low PH activates the viral M2 ion channel allowing protons to flow in facilitating the acid medium and causing the M1 proteins to detach from RNPs. The acidic medium also facilities the fusion of the HA with the viral envelope releasing the RNPs into the cytoplasm which are then rapidly imported into the nucleus of the host cell through the nuclear pore complex. This is thought to be facilitated by the viral PB2 and NP (Pleschka, 2012).

In the nucleus of the host cell the viral RNA polymerase carries out transcription using the 5'cap of host cell mRNA in a process called cap snatching of the viral RNA (vRNA) segments into viral messenger RNAs (mRNAs), also serving as a template for a complimentary copy (cRNA) intermediate. These positive RNAs form complementary RNPs (cRNPs) and act as templates for the production of new vRNAs. Viral mRNA is then forwarded to the cytoplasm for translation by cellular mechanisms (Pleschka, 2012).

Newly synthesized viral RNA polymerase subunits such as polymerase basic 1 (PB1), PB2 and polymerase acidic (PA) and nucleoprotein (NP) are then imported into the nucleus and bind to vRNA genomic segments and cRNAs to assemble vRNPs and cRNPs, respectively. Following nuclear export, progeny vRNPs are transported across the cytoplasm on recycling endosomes to the cell membrane, where progeny virions assemble (te Velthius, 2016).

These new mature virons incorporate a substantial amount of host proteins and are released from the cell membrane by budding with the help of receptor- cleaving NA proteins of IAV and IBV or the ICV HEF protein.

The replication process of the virus is demonstrated in **Figure 2**.

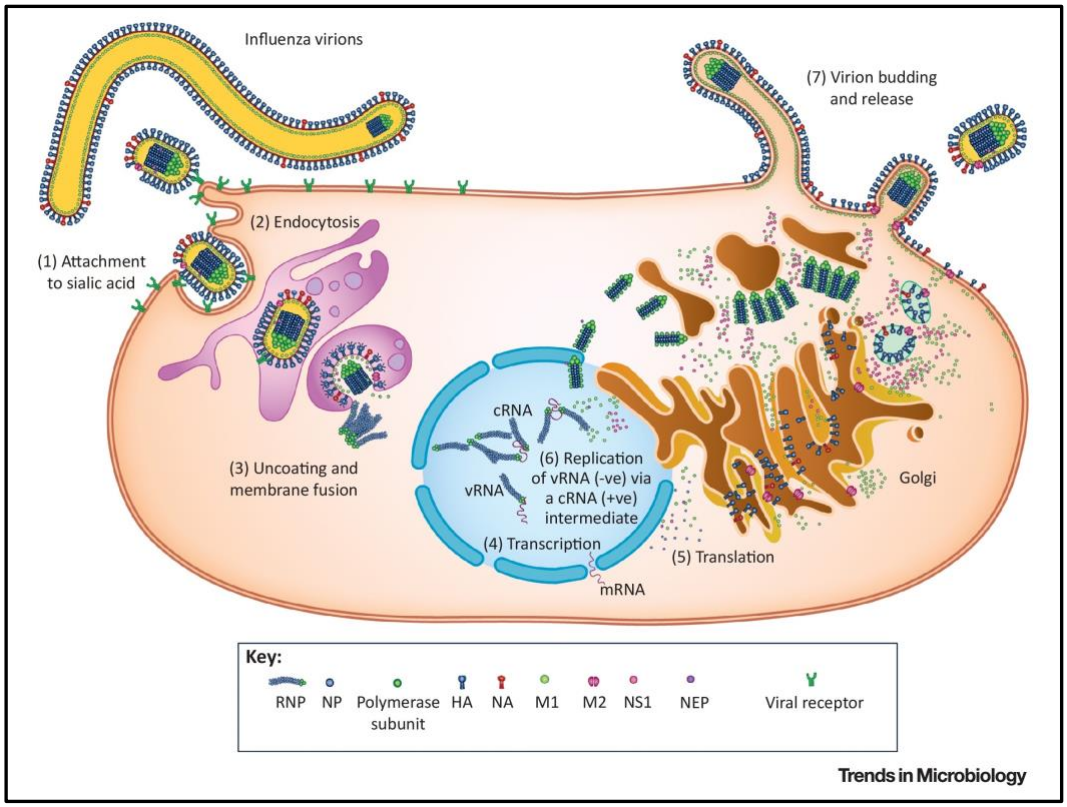

Figure 2. Replication of influenza viruses (Hutchinson, 2018).

The influenza virus is separately categorised based on the HA and NA subtypes. The segmented genome and the 18 HA and 11 NA subtypes assist in the high mutation and replication rates of the virus. Mutations of the virus lead to resistance to the current vaccines and medications as well as the immunity being bypassed. Any gradual change in antigenicity make up causing the current vaccines to be ineffective or less effective is called antigenic drift. This is the reason the human influenza vaccine composition is reviewed and changed every year (Pleschka, 2012).

The virus contains hypervariable genes that allow the possibility of antigenic drift, which is essentially point mutation of the genes of surface proteins HA and NA. The mutation happens during replication, after the RNPs are released from the virus membrane and infiltrated into the nucleus of the host cell, when the RNA polymerase transcription takes place there can be mistakes in the coding. If there are enough of these point mutations the host antibodies cannot recognise the antigen and a new subtype is created.

Antigenic shift is when there is a sudden complete change in the genome sequence due to genetic reassortment, which leads to a novel virus that can replicate in new types of host cells, often resulting in a pandemic. Antigenic shift occurs when two different strains of the influenza virus infect one host cell and during replication in the cell there is a transfer of

segments and a new strain is developed. Reassortment of similar viruses may occur frequently but is only noticeable if it occurs between two distinctly different co-circulating strains. In a report on antigenic and genetic variations of the EIV strain H3N8, analysis of H3N8 viruses showed reassortment of RNA segments encoding NP, PB2 and PA between the equine H7N7 and H3N8 subtypes (Bryant , et al., 2009). The difference between antigenic drift and shift is illustrated in **Figure 3**.

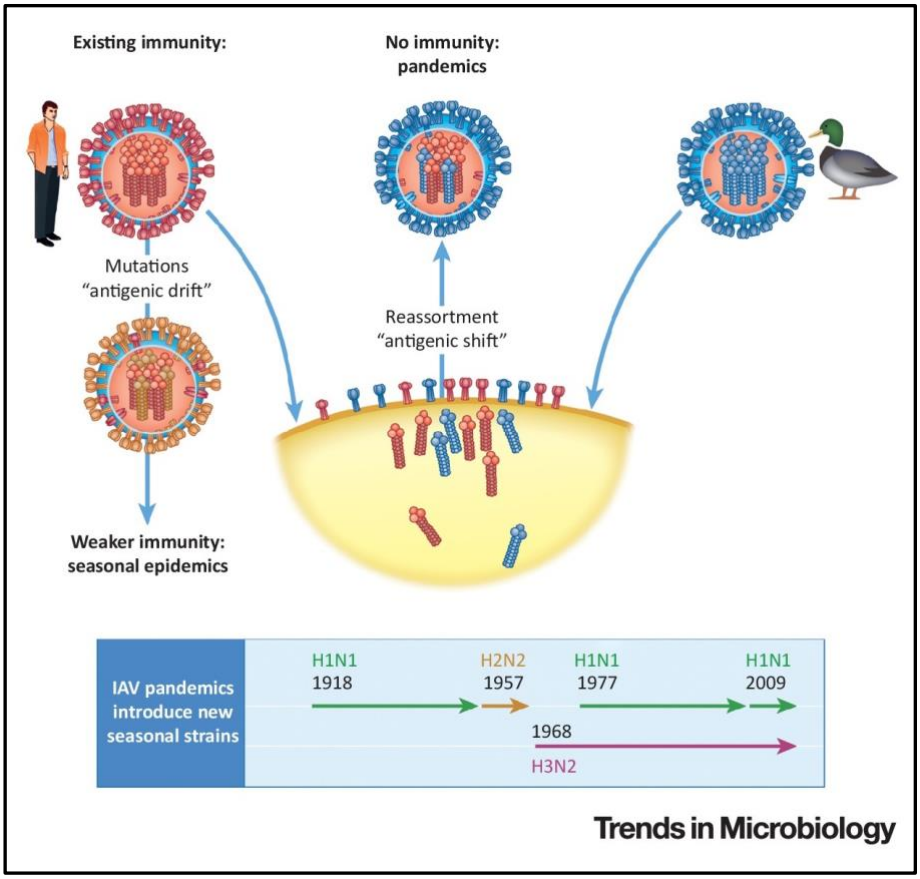

Figure 3. Difference between antigenic drift and antigenic shift (Hutchinson, 2018)

The HA subtype is a dominant determinant of tissue tropism, viral spread, and pathogenicity in influenza-infected organisms due to its antigenic properties, receptor binding and fusion activity. In humans, antigenic shift leading to a pandemic has only been recorded three times to date, the first being the Spanish flu in 1918, the second being the Asian flu in 1957, and third the Hong Kong flu in 1968.

The most recent pandemic in 2009 with the H1N1 type influenza virus of swine origin was a result of a combination of genome segments from humans, birds and swine. Highly pathogenic avian influenza strains also pose a threat to humans as seen in the 1997 Hong Kong outbreak, where a HPAI strain directly infected humans (Pleschka, 2012).

5. Equine influenza disease

Equine influenza is caused by EIV, which is an influenza A virus with 8 genome segments. It is a disease with high morbidity and low mortality; mostly affecting younger horses. In a fully susceptible animal the main clinical signs are coughing, nasal discharge, pyrexia, dyspnoea, anorexia and in very rare clinical cases ataxia (Bryant , et al., 2009).

Mortality is very rarely seen in animals infected with equine influenza but there has been a small number of fatalities reported in young foals that did not acquire maternal antibodies, and horses and donkeys that are not given adequate rest (Cullinane & Newton , 2013).

In an outbreak in North-eastern China in 1989 the causative agent was a H3N8 that was more closely genetically associated with avian influenza than other equine H3N8 viruses. This strain had a mortality rate of 20%. Fortunately, this strain did not appear to be identified after 1990 (Cullinane & Newton , 2013).

In unvaccinated or susceptible horses, the morbidity rate can be up to 100%, and this can be facilitated by the environment in which the horses are situated. An enclosed indoor housing area combined with animals with a persistent cough can greatly increase the risk of infection. The amount of clinical cases has been reported to peak at day 8 post primary infection in this type of environment (Cullinane & Newton , 2013).

EIV is a self-limiting disease and the virus does not persist in recovered animals. However, it is mentioned in a report that the virus persists in low levels by circulating with infrequent small outbreaks in endemic areas (Cullinane & Newton , 2013).

Foals usually obtain maternal antibodies within 48 hours of birth if they are born to seropositive mares. These antibodies last for three to six months, but in some cases for even longer. For this reason, vaccination should not be done before 6 months to avoid a decreased serological response from the vaccine due to maternal antibodies still being present. This was seen to be a risk factor of EIV infection in racecourses in Newmarket in 2003 (Cullinane & Newton , 2013).

6. Outbreaks in the past

The dispersion of horses following race meetings, horse shows, sales and other events where equine influenza virus has been circulating is seen as one of the contributing factors of spreading the virus to the wider equine population. This was seen in the UK in 1979 at the well-known show the Olympia and again in 1989 at the Royal Dublin Horse Show in Ireland, which appeared to be the source of the outbreak (Cullinane & Newton , 2013).

A significant outbreak was recorded in 1986 in South Africa, with the introduction of 6 horses from the USA. It was significant as South Africa had been previously free of the disease therefore the equine population was naïve to the virus. One of the 6 horses was noted to have influenza-like symptoms on route to Johannesburg such as nasal discharge, couching and listlessness. On arrival in Johannesburg the animal was examined by a private practitioner and the symptoms were put down to the long journey and fatigue (Guthrie, et al., 1999).

The animals were then placed in a quarantine building with 12 other horses, of which some were vaccinated and some were not. Vaccination was not a prerequisite to enter into South Africa at the time of the study. After two horses left the quarantine facility, cases of sick horses in many yards started to emerge (**Figure 4**). For example, approximately 320 of the 450 horses at Turffontein racecourse showed signs of a viral respiratory infection by the 15th of December 1986.

Nasopharyngeal swab samples were taken on the 12th of December 1986 at a yard belonging to a trainer who had been at Turffontein as equine influenza was suspected. EIV infection was confirmed by virus isolation, the method used was inoculation of 10-day old embryonated chicken eggs. Eggs were incubated at 35 degrees for 48 hours. The allantoic fluid was harvested and a haemagglutination test was carried out to confirm influenza infection (Guthrie, et al., 1999).

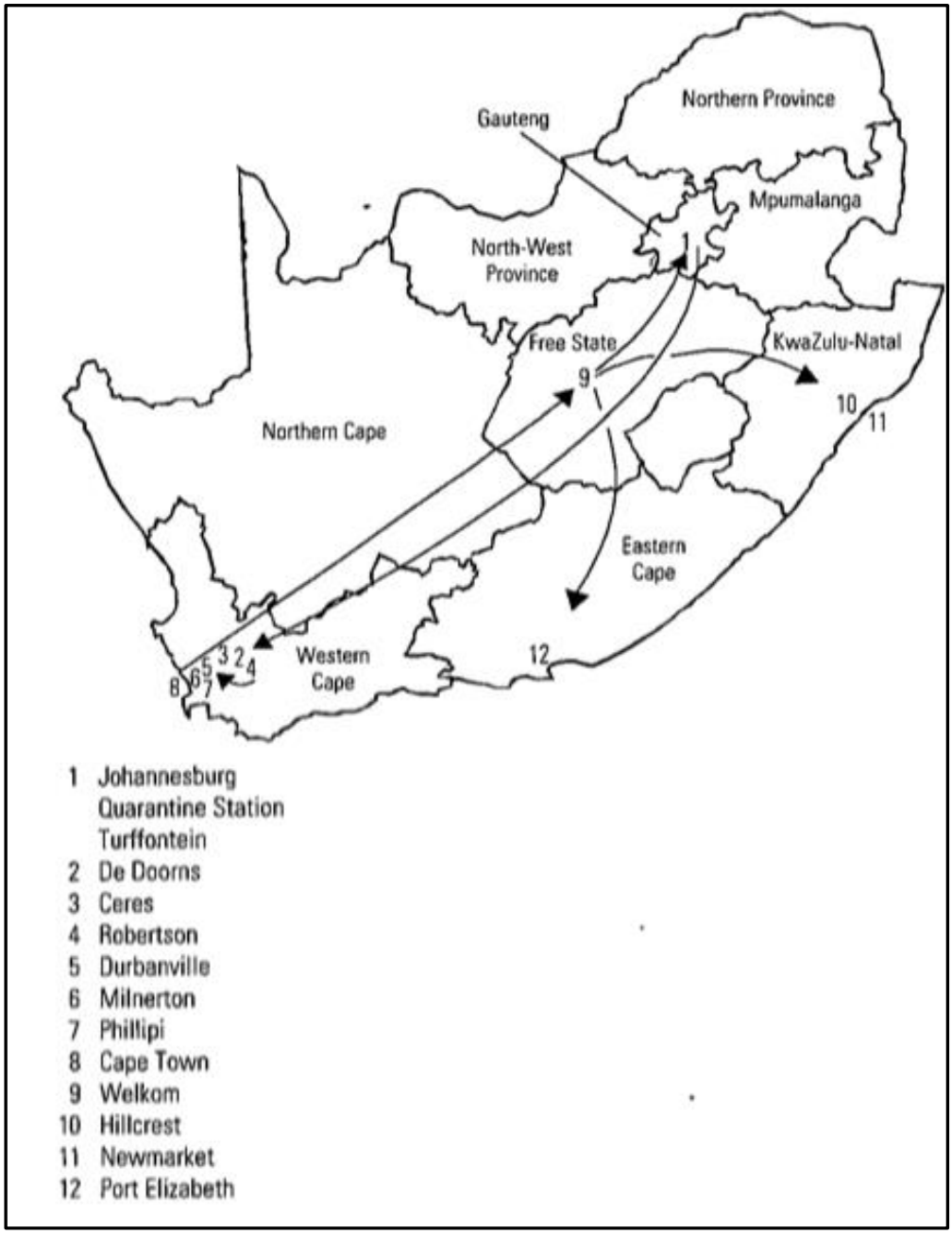

Figure 4. Map showing the distribution of individual outbreaks of equine influenza in South Africa between 10 and 17 December 1986 (Guthrie, et al., 1999)

Control measures were implemented immediately after confirmation of equine influenza. As South Africa was free of the disease until this point the disease was classified as a controlled disease. Movement restriction were implemented in the affected regions immediately, all race meetings in the regions were also cancelled. All equestrian events were urged to be cancelled but some did not comply.

A vaccination plan was put into place for all registered thoroughbreds, including a primary and a secondary vaccination. An inactivated equine influenza vaccine containing the

Johannesburg/86 strain was released in March 1987 and a bivalent vaccine also containing the influenza equi-1 antigen was released in July 1987.

Vaccination of all horses, donkeys and mules became compulsory and by the end of September 1987 the last case of equine influenza was detected. South Africa has been free of equine influenza since, proving that with the right implementations the virus can be effectively removed (Guthrie, et al., 1999).

Similarly, in 2007 there was an outbreak in Australia, which previously had an (OIE) free status for EIV. The outbreak was due to a breach in quarantine at the Eastern Creek Animal Quarantine Station. As the equine population in Australia was naïve to EIV there was a significant outbreak affecting over 76,000 animals. (Paillot & El- Hage, 2016)

The outbreak originated from horses imported from Japan that was experiencing an EIV outbreak at the time. The EIV strain affecting Australia was identified as A/equine/Sydney/2888-8/07(H3N8) which is a member of the FCL1 sublineage. (Paillot & El- Hage, 2016)

The outbreak in Australia was first confirmed on the 24th of August 2007. With the help of testing and control protocol put in place by the government, Australia regained their EIV free status 12 months after the last detected case in December 2007.

The vaccination selected for the emergency vaccination programme was a live recombinant (canarypox) vectored vaccine containing two recombinant vector vaccines expressing haemagglutinin of the EIV strains A/equine/Newmarket/2/93-(H3N8) European lineage and A/equine/Kentucky/94(H3N8) American lineage. This vaccine was selected as the nucleoproteins allowed differentiation of infected animals to vaccinated animals (DIVA) (Paillot & El- Hage, 2016). The method of DIVA used in Australia at the time was a combination of haemaggluttination inhibition (HI) and NP-ELISA.

The vaccine encoded the EIV HA gene only, therefore seroconversion of immunised animals only affected the HA protein. The testing method is based on detection of antibodies that can prevent agglutination of erythrocytes. The concentration of antibodies is assessed by the highest serum dilution that inhibits agglutination (haemagglutination inhibition titre) (Wang, et al., 2017). The vaccine also contained NP protein, therefore the immune system naturally produced antibodies against this, allowing a specific ELISA test (NP-ELISA) to detect vaccinated animals.

Arguably single radial haemolysis (SRH) assay is a better choice than HI and NP-ELISA to measure antibody response in terms of reproducibility but it has a longer incubation period for results. Additionally, it is more labour intensive as it involves measuring the level of diffusion of antibodies in an agar gel to gauge the antibody content of test sera. This method quantifies antibodies by measuring the areas of haemolysis (Wang, et al., 2017).

The recombinant canarypox vector vaccine did not have the full criteria outlined by OIE recommendations on EI vaccination strains but at the time no commercial vaccine did.

The emergency vaccination plan implemented involved vaccination of all susceptible animals in a 10km wide vaccination zone, this was referred to as ring vaccination. The plan also included predictive vaccination and blanket vaccination (Paillot & El- Hage, 2016). Predictive vaccination concerned the vaccination of all animals that would have an economical affect such as thoroughbreds and police horses. Blanket vaccination involved vaccination of all susceptible animals to maximise the immunity in a specific area.

Due to the emergency protocol and vaccination plan implemented in Australia, the OIE free status was reclaimed in December 2008 (Paillot & El- Hage, 2016).

There is a global initiative on sharing all influenza-related genetic data through GenBank that contains each of the sub-lineages of EIV, this consists of Pre-divergent, Eurasian, American and Florida clades 1 and 2. They are separated according to their nucleotide and amino acid sequences and compared using phylogenic analysis (**Figure 5**).

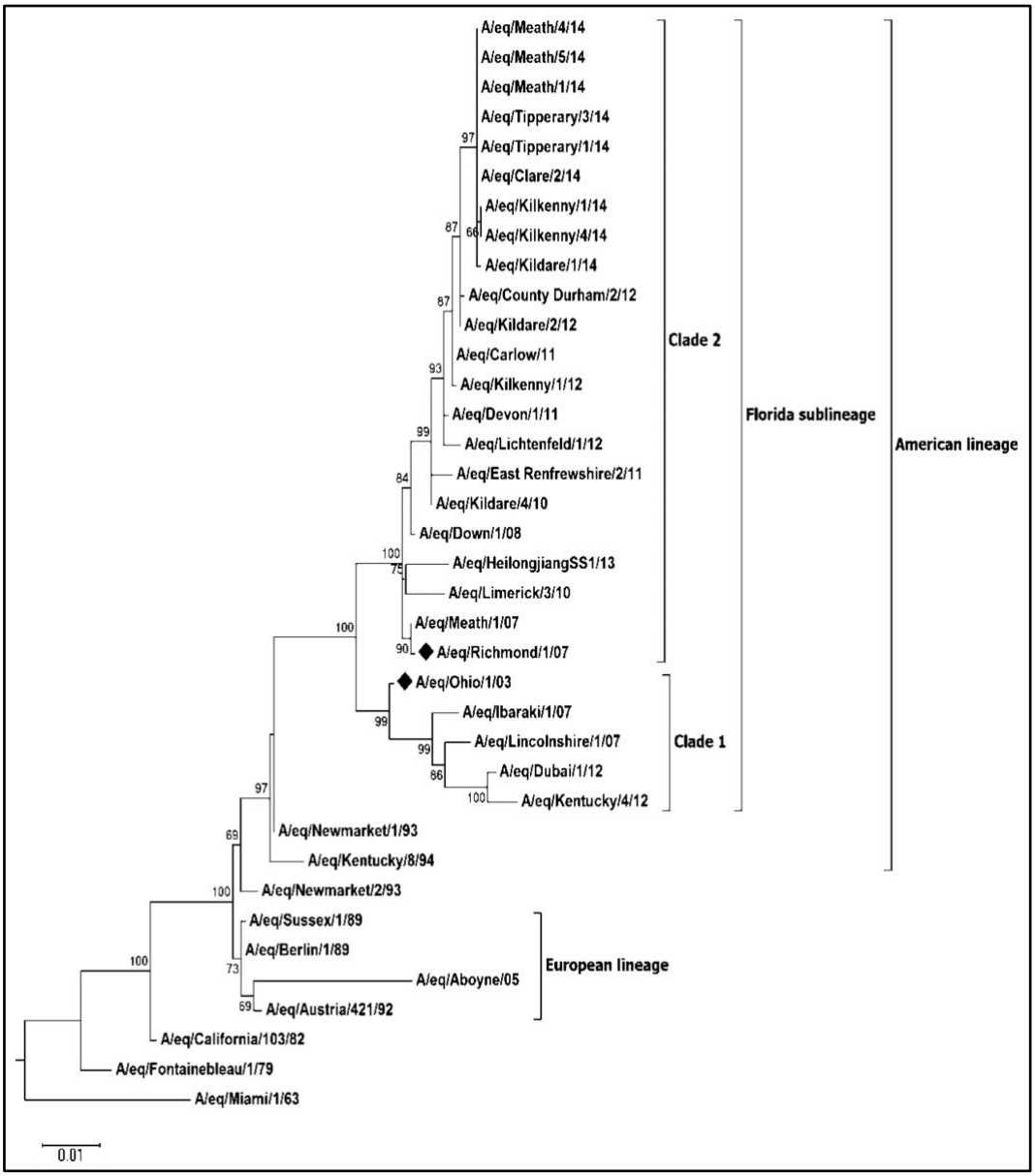

Figure 5. Representation of phylogenetic analysis of NA nucleotide sequences encoded by equine influenza viruses. (Gildea , et al., 2018)

During the 2015 Malaysian outbreak and as with all outbreaks of EIV the virus was isolated from infected horses. A nasopharyngeal sample was taken from 25 thoroughbred horses transported from Malaysia to Singapore with influenza-like clinical signs, and RT-PCR targeting the influenza A matrix proteins and a rapid test were used to confirm EIV. These samples were then cultured in SPF embryonated eggs and the result were confirmed using haemagglutination assay and RT-PCR (Wang, et al., 2017).

The genetic sequences of the HA and NA were confirmed using Sanger sequencing. Sanger sequencing involves separating the genetic sequences with automated gel electrophoresis and fluoreseent terminator chemistry (Mcginn & Glynne Gut, 2012).

Phylogenic analysis was then used to compare the nucleotide and amino acid sequences of the HA and NA gene segments (Wang, et al., 2017).

All HA sequences were identified within the FCL1 sub-lineage, suggesting that H3N8 caused the clinical manifestation in the horses tested. Two genetic variants were identified (A/eq/Malaysia/M201-1/2015) and (A/eq/Malaysia/M201-2/2015).

When compared to the FCL1 strains, there was eight other amino acid substitutions identified. Some of these amino acid substitutions were seen at antigenic sites. This adds to the theory of continued evolution of the virus and antigenic shift which may have led to the inefficiency of the vaccine as all 25 horses tested were said to have been vaccinated.

Currently it is unknown how many amino acid substitutes are needed to effect the vaccination efficacy, but this outbreak and the isolation and sequencing reported in the paper of (Wang, et al., 2017) proves the need for continuous examination of the genetic sequences of emerging EIV strains.

7. Current (2019) outbreak

The first incidences of most recent EIV outbreak in the UK were diagnosed in late 2018, early January 2019 with some of the first identified outbreaks occurring in Essex, Cheshire and Derbyshire.

Overall in the UK there were 228 laboratory confirmed outbreaks in 2019 with more than 15,000 samples screened. There was likely to be many more cases that went undetected, which may have been due to insufficient testing or reporting by owners. The causative agent was identified as a strain of the FCL1 of the H3N8 equine influenza virus.

This was the first time that a clade 1 virus, which is primary dominant in North America had caused multiple outbreaks in the UK since 2009 and in Ireland since 2010 (International collating centre, n.d.) . It was also the first time since the 1980s that races had been cancelled due to EIV. The racing events throughout the UK were closed for a total of 6 days, from February 7th to 13th with the media reporting that there was over £100 million in revenue loss (Cullinane , et al., 2020).

On the 18th of January 2019 the Irish Equine Centre issued a statement confirming outbreaks on five premises. It transpired that the first two premises, a thoroughbred stud farm and a show jumping yard had epidemiological links to Europe, with animals having recently travelled back from Europe (Irish equine centre , 2019). Both vaccinated and unvaccinated horses were testing positive for EIV; it was confirmed by the Irish equine centre which is an OIE reference laboratory. The virus was typed as a FCL1 virus by sequencing the HA and NA gene.

Not all commercial vaccinations available in the UK and Ireland contain the FCL1 strain. Only vaccines that contain A/eq/South Africa/04/2003 or A/eq/Ohio/2003 do. In EIV naïve horses, either who were vaccinated but lacked sufficient immunogenic protection or who were unvaccinated, the incubation period was seen to be a short as 24 hours.

In case of immunogenically protected animals the incubation period was up to 3 to 5 days and clinical signs were much less severe with some only showing a slight spike in temperature. This led to some cases going undiagnosed, allowing the virus to spread to a wider field (Irish equine centre , 2019).

Figure 5. shows the overall outbreaks within the UK from January 2019 to July 2020. The peak of the outbreaks was clearly seen in mid-summer.

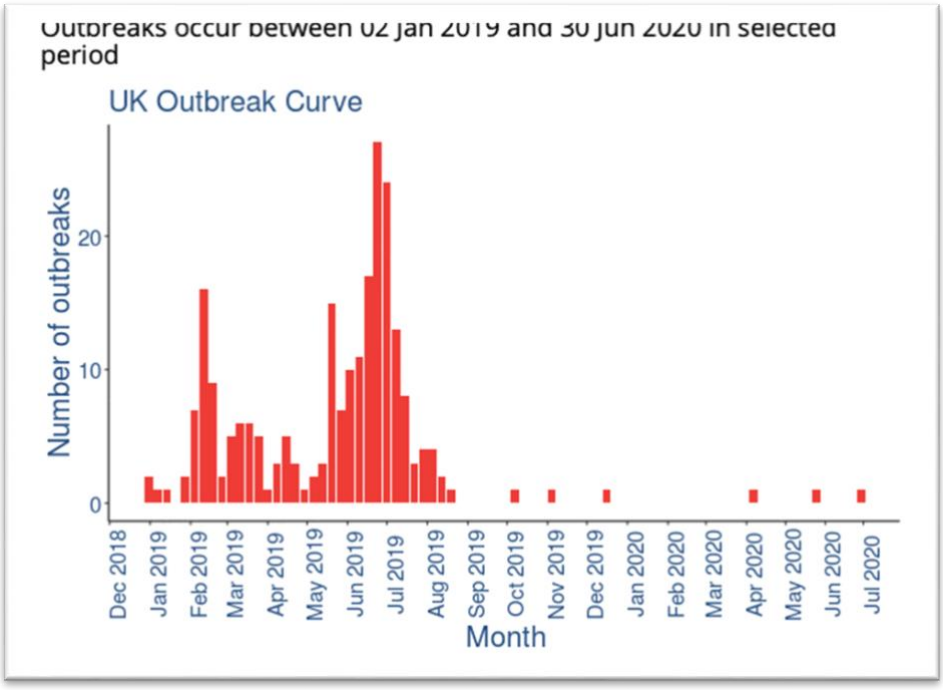

Figure. 5 Outbreaks in the UK occurring between January 2019 and June 2020. (BEVA, 2019)

Figure 6. reveals that the primary outbreaks occurred mainly in Essex, Cheshire, Derbyshire and Yorkshire, but later results from December 2019 highlight that the worst affected areas were in Durham (**Figure 7.**).

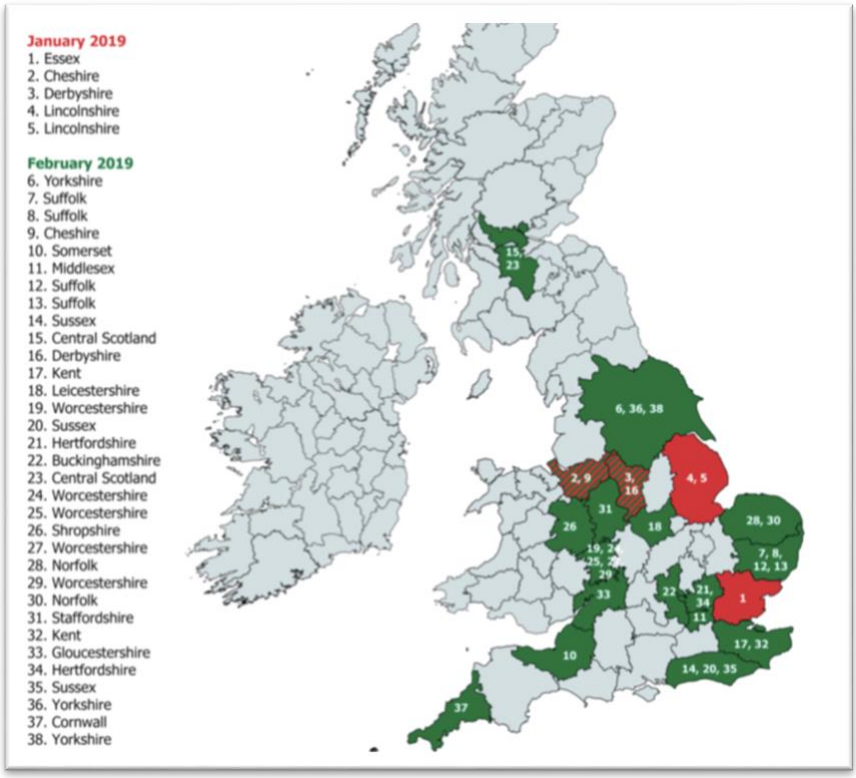

Figure 6. Primary outbreaks from January and February 2019 (DEFRA AHT BEVA , 2019)

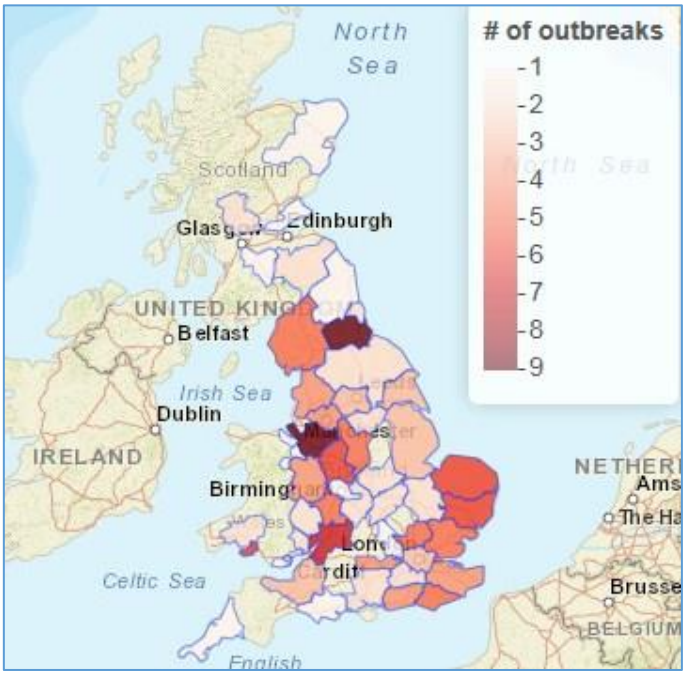

Figure.7 Number of outbreaks from January 2019 to June 2019 (BEVA, 2019)

Outbreak No.	Date	County	Vaccinated?*	Diagnosed by	Clade of virus
1	26/02/18	East Lothian	No	qPCR on NP swab	FC1
2	17/04/18	Oxfordshire	YES	qPCR on NP swab	ND

Figure 8. summary of cases of EIV in the UK in 2018, highlighted the diagnosis methods used in the 2019 epidemic (Equiflunet , 2019) (NP = nasopharyngeal, FC1 = H3N8 Florida sub-lineage clade 1.

Figure 8. is a summary of the confirmed outbreaks in 2018, showing the diagnosis methods used in the UK in the 2019 epidemic. For the diagnosis nasopharyngeal swabs were used, these can be used for the detection of EIV NP by qRT-PCR as it gives fast results.

According to (Galvin , et al., 2014) paper on evaluation of three diagnostic tests for the detection of equine influenza nucleoprotein in nasal swabs, DFA (direct fluorescent assay) or Directigen, which is a rapid antigen detection test is the most sensitive among the antigen detection methods. DFA can detect viruses in subclinical infected horses and vaccinated horses. The study also declares that DFA should be used in conjunction with other tests such as qRT-PCR as its sensitivity is only 68% when compared to the high sensitivity of RT-PCR considered to be the gold standard (Galvin , et al., 2014).

RT-PCR is the most sensitive technique for laboratory diagnosis of equine influenza. RT-PCR has been seen to give positive results long after the infectious phase of 14 days has passed, with RNA being detected up to 34 days' post infection. It may provide positive results when all other diagnostic tests are negative (Galvin , et al., 2014).

These results explain why the Animal Health Trust chose to use both rapid antigen detection tests and RT-PCR to test for EIV cases.

The OIE also reported EIV outbreaks spreading across Europe in 2019 (**Figure 9.**), appearing to peak in late summer to Autumn; mainly seen in UK, Ireland, France, Sweden, Belgium, Germany, and the Netherlands (OIE, 2020). Among these countries, UK and Ireland had the highest number of cases.

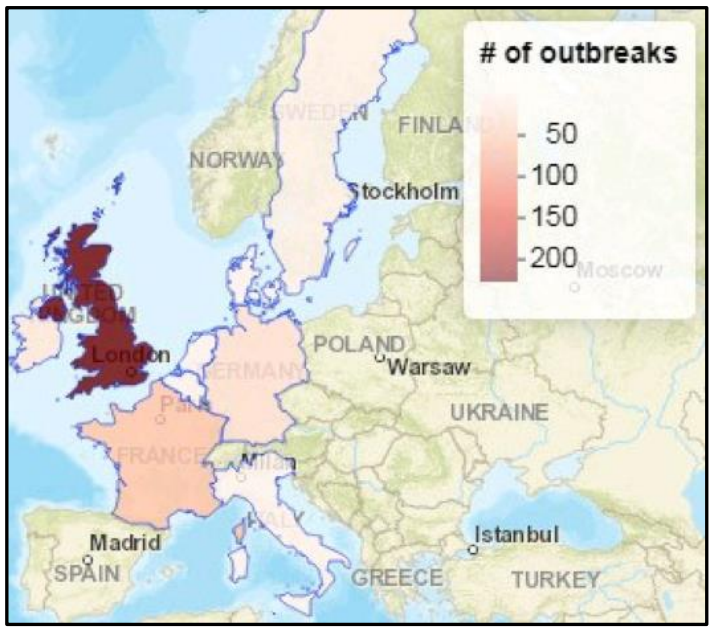

Figure 9. Outbreaks in Europe from 1 January to 11 October 2019 (DEFRA AHT BEVA , 2019)

8. EIV vaccination

8.1 Types of vaccines

Vaccination against EIV became mandatory in all racehorses in the early 1980s in the UK, France and Ireland after a huge outbreak in 1979 that led to the cancellation of many races throughout each of these countries and led to significant financial losses. Vaccination ruling and protocols are put in place to lower the economic impact of the disease. The vaccines available decrease the virus shedding and clinical signs caused by the virus but cannot always prevent infection. The vaccine creates a humoral response to the virus and produces antibodies against the envelope glycoproteins that has been indicated to protect against EIV (Entenfellner, et al., 2020).

There are currently three main types of equine influenza vaccine in circulation worldwide:

- The canary pox recombinant vaccine, which contains a representative from the FCL1 strain and one from the FCL2 strain. These have been inserted into two canary pox vector vaccines. This is the only vaccination type that adheres to the OIE recommendations.

- The inactivated vaccine provides active immunisation against equine influenza using the whole influenza virus and contains a representative from the European lineage and the American lineage.
- The immune stimulating complex-based subunit vaccine contains three equine influenza strains, a representative from H7N7, which is no longer recommended by the OIE as the strain has become obsolete, a representative from H3H8 European and a representative from the American lineage.

8.2 Efficacy of vaccine

When testing the efficacy of the vaccination single radial haemolysis (SRH) is the diagnostic tool of choice. SRH assay analyses and compares antibody production against different strains of the virus (Paillot, et al., 2013). This method quantifies antibodies by measuring the areas of haemolysis. SRH, when tested with untreated antigens has been seen to be more reproducible between laboratories and has shown a strong correlation between SRH antibody levels and protection from vaccines (Gildea , et al., 2018). SRH antibody titres have become established analogues of protection. Although HI can also be used, SRH is the only test that is standardised internationally with reference sera available from the European Directorate for the quality of medicines (EDQM).

Protection against infection of the virus also known as virological protection can be quantified as antibodies titres >150mm^2, if the vaccine strain is closely linked to the epidemic virus strain. Antibody titres >85mm^2 is connected to clinical protection but the horse may still shed the virus (Entenfellner, et al., 2020).

Strain-specific antibodies are more effective than cross-reacting antibodies. Higher levels of antibodies are needed if there is a mismatch between the vaccine strain and the circulating strain to ensure protection. A representation of this is the 1987 EIV outbreak in South Africa, where the animals had no natural immunity to the virus as the country had previously been free from the disease. The strains of the vaccines were not homologous to the strain causing the infection, but the vaccines induced SRH levels of >165mm^2 that correlated with 90% seroconversion and all of them induced protective cross-reacting antibodies. Therefore, besides the intricacy of heterogenic strains, SRH is known as the gold standard when comparing protectiveness of vaccines (Cullinane , et al., 2020).

In (Entenfellner, et al., 2020) study, SRH was one of the diagnostic tests used to evaluate the efficacy of the different vaccine types on young sports horses' six month booster vaccine. The results showed a significantly lower antibody response to the subunit and recombinant canary-pox vaccines when tested against the whole inactivated vaccine. There was also a delayed antibody response from the recombinant vaccine. The recombinant canary-pox vaccine is the one that contains the strains recommended by the OIE, while also allowing for differentiation vaccinated animals to infected animals (DIVA) therefore it is important to allow time for the antibody response (Cullinane , et al., 2020).

For the first 3 months post vaccination there was a lower antibody level from the recombinant vaccine when compared to the inactivated and the subunit type (**Figure 10.**). 3 to 6 months post vaccination there was no significant difference in the performance of the 3 vaccines (Entenfellner, et al., 2020).

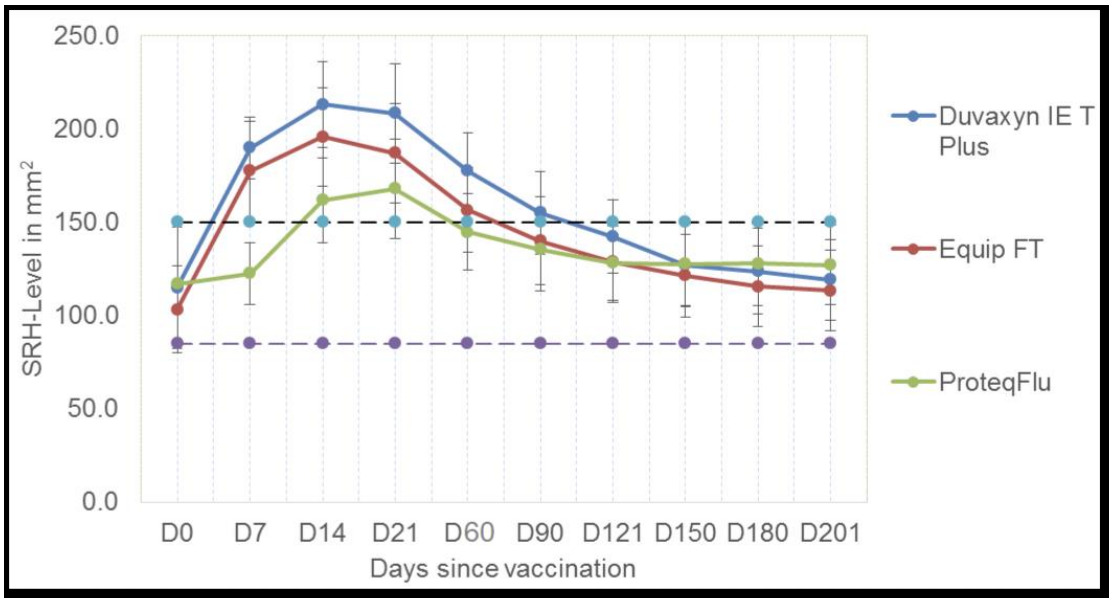

Figure 10. The SRH levels tested against three of the commercially available vaccines. ProteqFlu-Te: recombinant canary pox based vaccine; Equip FT: subunit vaccine; Duvaxyn IE-T Plus: inactivated vaccine. Blue horizontal dashed line: threshold level of SRH associated with virological protection; purple horizontal dashed line: threshold level of SRH antibodies associated with clinical protection (Entenfellner, et al., 2020)

8.3 Racing industry vaccine recommendations

The recommended dosing protocol is of significant importance in ensuring the complete protection and antibody production against the virus. A study of Ann Cullinane (Cullinane & Gildea, 2013) showed that with just the primary V1 dose of the vaccine there was a mean antibody response of clinical protection but not of virological protection, proving there is a need for the second dose and booster vaccine. It also resulted in showing that lengthening the vaccination interval increased the immunity gap but did not lower the response to the second and third dose. There was a similar response to V2 and V3 in all three regimes used during the study. Overall, there was less immunological protection in horses vaccinated with the maximum time intervals allowed by the racing industry compared with horses vaccinated with the minimal time allowed or vaccinated under the manufacturer's instructions (Cullinane & Gildea, 2013). The recommended dosing includes the 1st vaccination from 6 months, the 2nd vaccination 4 weeks after the first, the 3rd vaccination 5 months after the second, followed by an annual booster.

The racing industry have strict protocols on the vaccination regime. A primary course of 2 doses (V1 and V2) is required no less than 21 days and no more than 92 days apart. A booster (V3) is required no less than 150 and no more than 215 days after the second vaccine. This protocol is the same for the Jock Club in the UK, the Turf Club in Ireland, and Gallop France (Cullinane & Gildea, 2013).

8.4 OIE Recommendations

General vaccination programmes for EIV in endemic areas such as Europe and North America are based on recommendations by the OIE, the World Organisation for Animal Health. According to the recommendations as of April 2020, it is not necessary to include an H7N7 virus or an H3N8 virus of the Eurasian lineage in vaccines as these viruses have not been detected in the course of many years of surveillance and are therefore presumed not to be circulating.

Vaccines should contain both clade 1 and clade 2 viruses of the Florida sublineage. Clade 1 is represented by A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses and more recent clade 1 viruses are available from the OIE reference laboratories. Clade 2 is represented by A/eq/Richmond/1/2007-like viruses and more recent clade 2 viruses are

available from the OIE reference laboratories (OIE, 2020). Each country has a reference laboratory that works together with the vaccine manufacturing companies to produce vaccinations that are immunogenically reactive to the equine influenza virus that is circulating nationally.

These are the worldwide recommendations in place but unfortunately, they are usually not adhered to by the vaccine producers. For example, in the paper of (Bryant , et al., 2009) on antigenic and genetic variations it is stated that most commercial vaccines do not meet the current EIV strain recommendations and would require higher amounts of SRH antibodies for protection against clinical signs of the disease and virus shedding of strains genetically different from vaccine strains. This is further confirmed by (Gildea , et al., 2018), stating that only one commercial vaccine, an updated ProtectFlu recombinant vaccine containing a representative gene sequence from FCL1 and FLC2 became available in Ireland in 2014.

Currently the recommendations regarding the booster before competition or transport are different for the OIE, FEI and IFHA. The OIE recommends immunisation between 21 and 90 days before shipment, whereas the IFHA recommends vaccination during the 60 days immediately prior to export from the country of origin but not within 14 days of export. The FEI recommends competing horses to be given their last booster within 6 months + 21 days and not with 7 days before arrival at the event (Cullinane , et al., 2020).

9. Current Vaccination

9.1 Vaccines currently used in the UK and Ireland

There are currently four equine influenza vaccines commercially available in the UK and Ireland (**Table 1.**).

- ProteqFlu-Te is a recombinant canary-pox based vaccine, contains parts of two equine influenza genes expressing the HA of A/eq/Ohio/03 (FCL1) and A/eq/Richmond/1/07 (FCL2), which have been inserted into two canarypox vector viruses respectively (Gildea , et al., 2018), and a tetanus toxoid that is a chemically weakened toxin from *Clostridium tetani*.
- Equilis Prequenza is an inactivated subunit vaccine, which provides active immunisation against equine influenza using the whole influenza virus and contains

the strains A/equine-2/South Africa/4/03, A/equine-2/Newmarket/2/93, and tetanus toxoid.

- Duvaxyn IE-T Plus is a whole inactivated EI vaccine that contains the strains A/eq/Prague/56 (H7N7), A/eq/Suffolk/89 (H3N8) and A/eq/Newmarket/1/93 (H3N8) (Paillot, et al., 2013).
- Equip FT is an immune-stimulating complex-based subunit vaccine containing three equine influenza strains, A/eq/Newmarket/77 (H7N7), A/eq/Kentucky /98 (H3H8) and A/eq/Borlange/91. It also contains tetanus toxoid.

Table 1.: UK vaccinations, compared to the OIE recommendations

Vaccine name	Conform to 1995 recommendations	Conform to 2004 recommendations	Conform to 2010 recommendations
Equip F	✓	✗	✗
Equilis Prequenza	✗	✓	✗
ProteqFlu	✗	✗	✓

9.2 Current vaccination protocols

Currently the aim is to work towards an evidence-based regime of vaccination protocol to ensure maximum protection against EIV.

In 2019, the Irish Horse Racing Board (IHRB) announced that from the 1st of January 2020 there would be new requirements regarding the equine influenza vaccination protocol.

The new rulings were to follow the recommendations made by the European Horserace Scientific Liaison Committee (EHSLC). The EHSLC is a professional body that is responsible for recommending harmonised policies related to veterinary matters, anti-doping and medication control across certain European countries such as Ireland, UK, France, Germany, Italyand the Nordic countries.

The objective of the EHSLC recommendation on this vaccination programme was to harmonize the policies across Europe to ensure maximum immunity while also considering the scheduling and practicalities of training racehorses.

The approach recommended by the EHSLC that was approved by the directors of the IHRB is as follows:

- The current rulings will apply in Ireland from the 1st of January to the 31st of December 2020. These state that to be eligible to run in Ireland the animals must have their initial vaccine (V1), then their 2nd vaccine (V2) 21-92 days following initial, and their 3rd (V3) 150-215 days after (V2). Their booster vaccine must then be administered at annual intervals (IHRB , 2019).
- The new ruling coming into place from January 1st, 2021 states that after the initial vaccine (V1), the second vaccine (V2) must be given within 21-60 days after initial vaccine, while the 3rd (V3) given within 120-180 days after (V2). These are much smaller intervals than those previously mentioned and may have been adjusted after the report of (Cullinane & Gildea, 2013) proving that the intervals of the initial 3 vaccination are of great importance. It concluded that lengthening the vaccination interval to the maximum allowed by the Irish racing industry would lead to an increased period of susceptibility to EI, although it must be mentioned that the study was only done using the subunit immune-stimulating complex vaccine (Equip FT), which was the vaccine routinely used by the veterinary surgeons at the time of the study.
- Finally, the booster vaccine must be given within the preceding 6 months, and 6 monthly intervals thereafter. The IHRB also recommends that all horses on the premises are vaccinated as spill over from unvaccinated non-TB horses are a large factor in the spread of EIV (Gildea , et al., 2018). Furthermore, that young horses are vaccinated as early as possible after six months of age. The vaccine should contain a Clade 1 representative (IHRB , 2019) as this was the responsible agent for the outbreak in the UK and Ireland in early 2019.

Since early 2019, the BHA, and the British Equestrian Federation followed the advice of the Animal Health Trust and other veterinary committees that a 6 month booster rather than annual boosters is immunogenically preferable (DEFRA AHT BEVA , 2019).

Prior to the 2019 outbreak, the BHA's cross-industry Veterinary Committee – in conjunction with the British Equine Veterinary Association (BEVA) had a protocol stating that the booster vaccination must be within a 12-month interval and every 12 months periodically after that to be eligible to race. After the outbreak, this period was shortened to 9 months to

decrease the period of time that the antibody titre was lower, which could lead to increased susceptibility to EIV (British racing authority , 2020).

During the early stages of the COVID-19 pandemic, BHA stated that due to the movement restrictions and the fact that there was no registered cases of equine influenza, the previous ruling of a 12-month interval on the booster vaccine was acceptable to be eligible to race, if the 9-month interval could not be adhered to.

The BHA policy that vaccinations must have taken place within the last nine months was an interim policy put in place at the start of 2019 with the view to implementing a permanent protocol, which was meant to be discussed and consulted on in 2020, but due to the COVID-19 outbreak this has been delayed (British racing authority , 2020).

10. Conclusion

Vaccines against EIV have limited protection and have to be repeatedly given because of the constant antigenic variations, in this case due to antigenic drift and shift of the HA and NA envelope glycoproteins of influenza virus strains (te Velthius, 2016). Evidence of this is seen in the report of (Toh , et al., 2019), where the HA amino acid sequences were compared to the clade 1 and clade 2 strains. There were 8 substitution amino acids in the 2015 Malaysia isolates compared to the FCL1 strain, suggesting the continuing evolution of this virus. Also the fact that some of the substitution amino acids were found on the antigenic sites indicates that there may have been an effect on vaccination efficacy (Toh , et al., 2019).

On the 18th of January 2019 the Irish equine centre issued a statement confirming outbreaks on five separate premises, with the first two of the premises, a thoroughbred stud farm and a show jumping yard having epidemiological links to Europe (Irish equine centre , 2019), indicating a link to travel and movement of animals as a factor in the spread.

In the study of (Cullinane , et al., 2020) on the current EIV vaccination protocols prior to shipment, they report two trials. Trial 1 determined the time necessary for the horse to respond to the booster vaccination; furthermore, how close to travelling could they be transported. Trial 2 determined how long the horse could be vaccinated before shipment while still maintaining sufficient levels of antibodies. As mentioned above the OIE, IFHA and the FEI currently have different recommendation on booster vaccination for EIV before transport. The study was done to evaluate a scientific-based protocol that would decrease

the risk of EIV outbreaks due to transportation. The study included horses from both the racing and sports horse industry, of a variety of ages, and vaccines from both international and national companies were used.

The results from the trial indicated that 14 days was adequate time for the antibody response to develop having received a booster vaccine. These results coincided with the requirements from the IFHA and OIE but not the FEI, which may have led to a higher risk of the spread of EIV in the 2019 outbreak through transportation of sports horses from Europe. The results also indicated that it is acceptable to vaccinate within 180 days of transportation if the animal is above 4 years of age or previously received four or more doses of vaccine. If the horse is four years old or younger, the results suggested that the animal would benefit from receiving its booster vaccine within 90 days of shipment, which is again within the OIE and IFHA recommendation but not that of FEI (Cullinane , et al., 2020). These findings would lead to the thought that if the different governing bodies could harmonize a protocol for vaccination before shipment, they could lower the risk of infection and spread of the virus.

All racing animals and FEI-competing animals have strict vaccination protocols that are under the control of the IFHA and the FEI respectively, but the majority of pleasure horses in Ireland are unvaccinated. This has been seen to be a source of infection in the past, with vaccinated animals coming from premises where there are many unvaccinated animals. In the 2014 outbreak in Ireland only 9% of the 159 non-TB animals involved in the study had up-to-date vaccination (Gildea , et al., 2018).

It has also been seen that the congregation of horses at any event such as hunts is facilitative to the virus. In the same study (Gildea , et al., 2018) done on the 2014 outbreak in Ireland, in 11 out of 18 of the premises involved the outbreak occurred following attendance at such events.

The timing of the three initial vaccinations is of high importance as mentioned above. In the study of (Cullinane & Gildea, 2013), it is claimed that the longest interval allowed by the turf club between V1, V2 and V3 leads to a period of lower antibody titre needed for protection against EIV and can lead to a higher risk of infection (Cullinane & Gildea, 2013).

The vaccines themselves were tested and the recombinant vaccine takes a longer time to produce antibodies, but is also the only vaccination with both FCL1 and FCL2 strains, which are part of the OIE recommendation. Consequently, the other vaccines may not provide sufficient protection against circulating strains as they only contain FCL1 strain.

Companies are not updating the vaccinations regularly enough to keep up-to-date with the constant antigenic drift, which leads to spread of the disease even among vaccinated horses (Gildea , et al., 2018). In the study done in Ireland during the 2014 outbreak, 31% of the vaccinated animals tested positive for EIV. The causative strain was identified as a strain of the FCL2 through genetic and antigenic analysis. Unfortunately, at the time of this study there was no commercially available vaccine containing the FCL2 strain (Gildea , et al., 2018).

An increasing problem with human medicine is certain people's willingness to vaccinate. Lack of confidence in vaccination can now be seen as a major threat to a successful vaccination programme. This is known as vaccination hesitancy, and it is also thought to be an increasing risk factor of vaccine-preventable disease outbreaks and epidemics (Dube , 2013) . In 2019, vaccination hesitancy was identified as one the top ten global health threats (Cullinane , et al., 2020).

This vaccine hesitancy has also begun to appear in animal medicine, with owners unwilling to vaccinate their animals as they are worried about the adverse effects of continuously frequent vaccination. In order for the vaccination programme against equine influenza to work there should be no vaccination hesitancy and in order for that to happen there must be confidence in the industry and the vaccine effect (Cullinane , et al., 2020). This can be improved through ensuring scientific-based vaccination protocols are in place and that the vaccinations are up to the current OIE standards, and contain the currently circulating strains.

From this theoretic literature review I can conclude that there is significant amount of research and literature on the topic of EIV that allows for the understanding in detail of the topic and the work that is being done worldwide to combat the disease. There is a large amount of research on the different outbreaks throughout the years and the different vaccination and testing protocols in place at the time of each. The research being carried out in the recent year being put towards more scientific-based protocols for vaccination programmes is a step forward towards contesting EIV. The most recent outbreak in 2019 has reinforced the importance of continued surveillance of EIV to assist in the identification of any new emerging strains of EIV. Ideally new variations or strains of EIV could be identified before leading to infection and outbreaks to avoid causing a threat to the equine industry.

11. Future possibilities

Consensus-based approaches and design of computationally-optimized broadly reactive antigens (COBRA) is a relatively new programme that begins with interfering the phylogenetic tree created from the HA amino acid sequences that are generated from each outbreak.

The consecutive subclade sequence is then generated from the primary sequence input. Finally, the secondary consensus is aligned and the resulting consensus sequences designated COBRA are generated.

Pre-clinical tests with vaccinated animal models are then done to determine the breadth of protection of the immune response against the novel antigen. This is part of the strategic plan of the US National Institute of Allergy and Infectious Diseases to produce a universal or broadly reactive human influenza vaccine. COBRA have shown more promising results in comparison to current standard of care (SOC), which has ranges between 10 and 60% protection. There is reason to believe that COBRA HA immunogens could overcome the negative causes of immunodominace, therefore elicit response to multiple epitopes. If this is the progression forward in human medicine, it seems to be reasonable to implement it also in veterinary science. Furthermore, this could be a development in a broad spectrum vaccination against equine influenza as a whole (Sautto & Ross , 2019).

There have been further advances in our knowledge on the structural formation of the influenza virus itself and more specifically the structure of the RNA polymerase. In the review of (te Velthius, 2016), the discussion focuses on how new antiviral drugs may be developed by targeting different areas on the influenza virus RNA polymerase. For instance, the site of interaction with the C-terminal domain (CTD) of the cellular DNA-dependent RNA polymerase large subunit is currently unknown but could be a potential target for antiviral drugs. These are developments in human medicine but in the future they could be a potential way of progression in the treatment of equine influenza. Without complications, clinical signs are usually a cough and fever for a few days and full recovery within 2-3 weeks, as the disease is self-limiting. Secondary infection may lead persistent cough and permanent damage to the respiratory system. This is not ideal in elite competition or racing horses, therefore this is where antiviral medicine could be beneficial in equine medicine.

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He was extremely helpful and I feel like I have deepened my knowledge of the topic of the equine influenza virus and the vaccination challenges that are faced today by the equine industry.

14. Appendices

HuVetA

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- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*

Appendix 4. Supervisor counter-signature form

I hereby confirm that I am familiar with the content of the thesis entitled STUDY OF VACCINATIONS USED FOR THE 2019 EQUINE INFLUENZA OUTBREAK IN THE UK written by SARAH GALLAGHER (student name) which I deem suitable for submission and defence.

Date: Budapest, 17 day 11 month 2020 year

DR. MAROSI ANDRÁS

Supervisor name and signature

DEP. OF MICROBIOLOGY AND INF. DISEASES

Department