# UNIVERSITY of LIMERICK

## OLLSCOIL LUIMNIGH

# *A Comparison of Diagnostic Tests for the Diagnosis of Equine Influenza Virus Infections*

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# *Abstract*

The rapid diagnosis of Equine Influenza (EI) is critical to the implementation of control measures. In this study, two rapid antigen detection (RAD) kits and three ELISA kits were evaluated as diagnostic aids in the detection of EI. The Directigen Flu A, Espline Influenza A&B-N and the ID Screen Influenza A Antigen Capture ELISA were compared to the traditional 'gold standard' diagnostic tests, virus isolation (VI) and reverse transcription polymerase chain reaction (RT-PCR). A total of 75 nasopharyngeal swabs were collected from horses on premises where natural outbreaks had occurred, and a total of 104 swabs were collected from experimentally challenged foals and tested by all five methods. RT-PCR was the most sensitive diagnostic technique. Directigen Flu A was the most sensitive kit in both sample groups detecting 46.95% positive in comparison to 16.18% (Espline Influenza A&B-N) and 16.96% (ID Screen Influenza A Antigen Capture ELISA). The superiority of Directigen Flu A was confirmed by comparing the limit of sensitivity of the three assays using known concentrations of virus. Post challenge, Directigen Flu A detected positives at the time of peak virus shedding but was less effective when horses were excreting low levels of virus. Thus Directigen Flu A test is a useful screening technique but negative samples from suspect cases should be submitted to a laboratory for testing by RT-PCR. The ID Screen Influenza A Antibody Competition ELISA which detects antibodies against the viral nucleoprotein (NP) was compared to the single radial haemolysis (SRH) test and the haemagglutination inhibition (HI) test. Paired samples from 203 horses on 14 infected premises were tested. Fewer seroconversions were detected by ID Screen Influenza A Antibody Competition ELISA (25%) than by SRH (43%) or HI (41%). The acute samples from the majority of affected horses that were not detected by ID Screen Influenza A Antibody Competition ELISA were seropositive. Post challenge in seronegative foals, the ID Screen Influenza A Antibody Competition ELISA detected a rise in antibodies earlier than the SRH test suggesting that in naïve populations this ELISA could provide a faster diagnosis than traditional serology methods. Sera collected from 60 weanlings following primary vaccination with five different vaccines (two whole

inactivated vaccines, two ISCOM based subunit vaccines and a canary pox recombinant vaccine) were tested by ID Screen Influenza A Antibody Competition ELISA and SRH. The ELISA did not detect the antibody response to vaccination with the canary pox recombinant vaccine (Proteq Flu Te), confirming the usefulness of the combination of this kit and vaccine to differentiate between naturally infected and vaccinated horses i.e. DIVA. The pattern of antibody response post vaccination detected with the ID Screen Influenza A Antibody Competition ELISA was similar to that detected by the SRH test for the other four vaccines. The antibody response to the other two subunit vaccines (Equip FT and Equilis Prequenza Te) was detected by ID Screen Influenza A Antibody Competition ELISA i.e. no DIVA capacity was evident. An ID Screen Influenza H7 Antibody Competition ELISA was also evaluated and demonstrated potential for measuring vaccinal response to H7N7 virus.

In summary the results of this study suggest that sensitive RADs and ELISAs may be useful supplementary tests in the diagnosis and management of EI and in the monitoring of vaccine performance.

# *List of Abbreviations*

| | |
|---|---|
| ANOVA | Analysis of Variance |
| cDNA | complementary Deoxyribonucleic Acid |
| CI | Confidence Interval |
| DIVA | Differentiation of Infected from Vaccinated Animal |
| EDQM | European Directorate for the Quality of Medicines |
| EHV | Equine Herpes Virus |
| EID | Egg Infectious Dose |
| ELISA | Enzyme Linked Immunosorbent Assay |
| EI | Equine Influenza |
| HA | Haemagglutination |
| HI | Haemagglutination Inhibition |
| ISCOM | Immune Stimulating Complex |
| MDA | Maternally Derived Antibodies |
| MDCK | Madin Darby Canine Kidney |
| NP | Nucleoprotein |
| OD | Optical Density |
| PBS | Phosphate Buffered Saline |
| PAQ | Post Arrival Quarantine |
| PEQ | Pre Export Quarantine |
| RAD | Rapid Antigen Detection |
| RNA | Ribonucleic Acid |
| RT-PCR | Reverse-Transcription Polymerase Chain Reaction |
| SE | Standard Error |
| SRH | Single Radial Haemolysis |
| TCID | Tissue Culture Infectious Dose |
| TMB | Tetramethylbenzidine |
| VI | Virus Isolation |
| VTM | Virus Transport Medium |

# *Author's Declaration*

I hereby declare that this MSc. is entirely my own work. It has not been submitted for any other academic award, or part thereof, at this or any other educational establishment.

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# 1 Introduction

Equine Influenza (EI) is a prevalent respiratory disease in the equine population. Subtype 2 (H3N8) EI is in circulation currently (Daly et al., 2011; Gildea et al., 2011a). It is a constant threat to the horse industry world-wide. In developing countries the impact of an outbreak of EI decreases the ability of work horses to carry out their daily tasks. In developed countries, outbreaks have frequently resulted in the cancellation of major equine sporting events in an effort to control the outbreak. In the case of the racing industry, this causes significant financial losses. The ability to identify the virus quickly is essential in order to manage the disease and the spread of infection (Daly et al., 2004, Gildea et al., 2011a). The international movement of horses by air transport is common practice now for competition and breeding purposes and has resulted in an increase in the incidence of influenza outbreaks worldwide (Powell, 1991).

This disease can spread rapidly among susceptible horses as the virus is transmitted by inhalation and is highly contagious (Powell, 1991, van Maanen and Cullinane, 2002). The persistent coughing and the short incubation period of 1 to 5 days contribute to the rapid spread of EI virus (van Maanen and Cullinane, 2002). Vaccination decreases clinical signs and virus shedding (Paillot et al., 2006). Traditionally diagnosis was confirmed by isolation of the virus or by the detection of a significant increase in antibody level between paired sera (Powell, 1991).These methods require laboratory expertise, are time consuming and expensive. It is imperative that sensitive and rapid diagnostic methods are available for the diagnosis of EI. A number of new test kits capable of rapid detection and also ELISA kits are now available and need to be evaluated in the field.

# 2 Literature Review

## 2.1 Epidemiology

The first confirmed outbreak of Equine Influenza (EI) was in Prague in 1956 (Influenza A/eq/1/Prague/56). This virus, subtype 1, was characterized as H7N7 (Sovinova et al., 1958). The subtype A/eq-1/H7N7 has not been isolated since 1979 and is generally considered to be extinct (Webster, 1993). In serological tests however, antibodies have been detected in unvaccinated horses (Madić et al., 1996). This suggests that the virus may be present in subclinical form (Mumford, 1990a; van Maanen and Cullinane, 2002; Daly et al., 2004).

In 1963, a second subtype of EI was isolated when an epidemic occurred at training centres in Florida in the United States. The second subtype of the virus was characterized as influenza-A/eq/Miami/63 H3N8 (Waddell et al., 1963). A/eq/H3N8 still remains in circulation today (Daly et al., 2011).

Widespread H3N8 influenza infection occurred in Europe and North America in both vaccinated and unvaccinated horses between 1978 and 1981 (Hinshaw et al., 1983; Klingeborn et al., 1980). The occurrence of infection in both vaccinated and unvaccinated horses provided the first evidence of antigenic variation. It also showed that antigenic variants co-circulate in equine populations (Hinshaw et al., 1983).

Subsequently, H3N8 viruses evolved into a Eurasian lineage and an American lineage (Daly et al., 1996, Daly et al., 2011). The 'American lineage' strains predominate currently and the 'Eurasian lineage' strains, represented by A/eq/Newmarket/2/93 have rarely been isolated in recent years. The 'American lineage' has diverged into three distinct sublineages, Florida, Kentucky and South America (Daly et al., 2011, Bryant et al., 2009). The 'Florida sublineage' has diverged into two clades. Clade 1 viruses predominate in North America but have caused outbreaks in South Africa (2003), Japan and Australia in 2007 and more recently in Europe (Bryant et al., 2009, Daly et al., 2011, Gildea et al., 2012). Clade 2 strains predominate in Europe and were also responsible for outbreaks in China, Mongolia and India in 2007 to 2009 (Qi et al., 2010, Virmani et al., 2010).

In China a highly lethal avian-like EI virus (H3N8) was isolated between 1989 -1990 and has not been isolated since (Guo et al., 1992; Lai et al., 2004). Two severe outbreaks occurred in Northern China in this period affecting over 20,000 horses. The virus was identified as A/eq/Jilin/89 (Lai et al., 2001).

In South Africa in 1986 and in India in 1987, major epidemics occurred in naïve populations (Daly et al., 2004; van Maanen and Cullinane, 2002). The source of these infections was traced to the importation of subclinically infected horses from countries where the virus was endemic. The analysis of the HA genes at the time confirmed the close relationship to the viruses circulating in the USA and Europe (Daly et al., 2004).

Further outbreaks were seen in Hong Kong in 1992 (Powell et al., 1995), Dubai in 1995 (Wernery et al., 1998), the Philippines in 1997 (Daly et al., 2004), and in Australia in 2007 (Cowled et al., 2009). These outbreaks were also attributed to the importation of subclinically infected horses that shed virus. These outbreaks highlight how easily an influenza virus outbreak can be introduced into susceptible populations and cause a major outbreak of disease (Daly et al., 2004).

The only countries with significant horse populations that have not experienced an outbreak are Iceland and New Zealand (Yamanaka et al., 2008). The immunologically naïve horses in a population are the most at risk of infection and the infection of a single horse can cause a rapid outbreak across a wide geographical area (Powell, 1991).

## 2.2 Disease and Clinical Signs

Equine Influenza virus epidemics occur most frequently in 2 and 3 year-old horses subsequent to races or shows. However, all naïve horses of all ages are susceptible to the influenza virus. The gathering of large number of horses in confined stables increases the chances of the virus spreading (Powell, 1991; van Maanen and Cullinane, 2002).

The incubation period for EI is approximately 1- 3 days. After this incubation, horses develop a fever that can reach 41°C, and can last 4-5 days (Paillot et al., 2006). Serous nasal discharge, tenderness in the mandibular lymph glands and a dry, non-productive explosive cough are typical clinical signs for this disease (Powell, 1991;

Wilson, 1993; Beech, 1991; Timoney, 1996). A variable degree of depression and anorexia can be seen as well as symptoms such as conjunctivitis, limb oedema, tachypnoea, tachycardia, dyspnoea and muscle stiffness (Timoney, 1996; van Maanen and Cullinane, 2002). However, in horses that are partially immune to the virus, clinical signs are less severe and cannot be distinguished from those associated with other respiratory diseases (Wilson, 1993; van Maanen and Cullinane, 2002). The most striking sign as noticed by van Maanen et al. (2003) after an outbreak in a riding school was a dry harsh cough. The sudden onset, frequent coughing and rapid spread of disease among all horses are the classical signs of influenza in susceptible horses (van Maanen and Cullinane, 2002).

Uncomplicated cases in the outbreak reviewed by Marois et al. (1963) only took a week to ten days for recovery, whereas cases with secondary infections, took from 10 to 21 days. The animal's age, previous exposure to the disease, environment and vaccination status are all factors that can cause the nature and severity of the clinical signs to differ from horse to horse (Timoney, 1996). As a result of fever, pregnant mares may abort the foetus. Foals are also susceptible and may rapidly develop fatal pneumonia. Donkeys are also more susceptible to influenza than horses (van Maanen and Cullinane, 2002).

Secondary bacterial infection can complicate the primary influenza infection. A continuous fever lasting longer than four to five days along with an abundant and viscous nasal discharge is an indication of secondary bacterial infection. Such bacterial infections include organisms such as *Streptococcus zooepidemicus* or *Pasturella /Actinobacilus* (Gerber, 1970; Timoney, 1996).


## 2.3 Transmission

Equine Influenza is a highly contagious respiratory disease, primarily transmitted by inhalation (Powell, 1991; van Maanen and Cullinane, 2002). Coughing contributes to the spread of influenza and the virus can travel at least 32 metres from an infected horse in an aerosolised droplet (Timoney, 1996). Partially immune and sub-clinically infected horses transported by air have caused transmission over long distances (Firestone et al., 2012). Contaminated fomites (such as buckets, twitches, head collars), substandard hygiene practices of stable and veterinary personnel and

contaminated transport vehicles can also play a role in the transmission of the virus to susceptible horses (Timoney 1996; Mumford et al., 1990a).

There is no evidence of a carrier state of EI in horses (Timoney, 1996). To reduce the spread of disease, good management practices, such as isolating the affected horses at the early stages of infection, should be in place.

## 2.4 Antigenic Drift

Antigenic drift is caused by the accumulation of mutations at the antigenic sites of the Haemagglutinin (HA) molecule which is the major surface protein of the influenza A virus (Bouvier and Palese, 2008; Daly et al., 1996). Antibodies that bind to the HA neutralize the infectivity of the virus. Virus strains evolve through amino acid changes at antigenic sites. This accumulation of mutations in multiple antigenic sites eventually disables the host antibodies ability to neutralize the virus, therefore leaving the host susceptible again to infection by the strain undergoing antigenic drift (Bouvier and Palese, 2008). Antigenic drift allows the virus to survive because the neutralizing antibodies for an earlier virus will not be able to protect the horse from the new mutated virus (Saito et al., 1993).

The understanding of antigenic drift is extremely important when considering the vaccination programme that needs to be in place to control the spread of infection. The efficacy of vaccines has been compromised regularly because of the antigenic drift of EI H3N8 strains. Regular updates of the vaccine strain are essential for the protection of the horses against the virus prevalent in the field (Cullinane et al., 2010).

An example of vaccine failure was seen in an outbreak that occurred in Croatia in 2004. The vaccine administered to the horses contained old strains of the H3N8 virus (A/eq/Miami/63, A/eq/Fontainebleau/79). Vaccinated horses showed the same clinical signs as horses that had not been vaccinated (Barbic et al., 2009). It is usually the situation that in vaccinated horses, the disease is subclinical, or there are fewer clinical signs for a shorter period of time than in unvaccinated horses (Barbic et al., 2009, Heldens et al., 2004). On comparison of the amino acid sequence of the virus in the Croatian outbreak, and that of the vaccine strains used, key differences at the HA1 subunit protein antigenic site were discovered, which probably accounted for vaccine failure (Barbic et al., 2009).

## 2.5 Vaccination

The H7N7 subtype of the EI virus subtype has not been detected recently but the H3N8 is still causing outbreaks and is not always successfully controlled by vaccination (Paillot et al., 2006). Many EI vaccines are whole inactivated virus vaccines and they stimulate short-lived humoral immunity. This is in contrast to the immunity that is stimulated by natural infection. Natural infection causes a longer lived and stronger immune response because both humoral and cellular immune responses are stimulated (Paillot et al., 2006).

The main aim of influenza vaccination is to provide clinical protection, leading to a shorter convalescent period and a reduced risk of secondary infections. The spread of infection should also be minimised by reduced shedding of the virus from infected horses. An effective vaccine should provide long-term immunity, a memory response that is efficient and should cross protect against different strains of the virus. It is estimated that 70% of the equine population should be vaccinated to prevent an influenza outbreak (Paillot et al., 2006). In 1980 vaccination against EI was made mandatory for Thoroughbred racehorses in the UK, France and Ireland. The vaccination programme required is as follows: a primary vaccination consisting of two doses $\geq$ 21 days and $\leq$ 92 days apart. The booster vaccination is then administered at $\geq$ 150 days and $\leq$ 215 days after the second vaccination. Horses must be vaccinated annually after this booster (Cullinane et al., 2001).

### Differentiation of Infected from Vaccinated Animals (DIVA)

Vaccination may, on occasion, be combined with an appropriate testing method to eradicate a disease by using a strategy to distinguish between the antibody response produced by natural infection and the antibody response produced by the vaccine (Uttenthal et al., 2010). This strategy is termed the DIVA strategy, as it involves Differentiation of Infected from Vaccinated Animals. This approach was taken with EI in Australia's outbreak in 2007 when horses were vaccinated with the canary pox recombinant vector vaccine Proteq Flu Te, (Merial) to minimise the spread of virus. This vaccine stimulates the production of antibodies to only one protein of the EI virus, namely HA. The canary pox vaccine was used concurrently with an ELISA test that detected the nucleoprotein (NP) of the virus in nasopharyngeal swabs so as to

allow identification and differentiation of vaccinated and naturally infected horses (Garner et al., 2011, Sergeant et al., 2009).

## 2.6 Diagnosis

### 2.6.1 Introduction

Equine Influenza was originally identified as a viral respiratory disease of the horse as a result of a number of unique features of the disease. These included the contagious nature of the disease, its close relationship with human influenza and the absence of secondary complications commonly associated with other viral respiratory diseases of the horse, such as equine arteritis virus and viral rhinopneumonitis (Marois et al., 1963). A clinical diagnosis can be confirmed in the laboratory using one of several different techniques. Diagnosis may be based on virus isolation (VI), serological response to the infection as demonstrated by a significant rise in antibody levels in paired serum samples collected approximately 2 weeks apart, or antigen or genome detection (OIE Manual, 2012). Samples required for the diagnosis of influenza include nasopharyngeal swabs for virus detection or clotted bloods for the detection of antibodies directed against the virus. Since an outbreak of EI can cause severe economic losses to the horse industry due to the cancellation of equestrian events and the implementation of movement restrictions, rapid diagnostic tests are required to facilitate the implementation of effective control measures. Table 2.1 summarises the main diagnostic methods for EI.

### 2.6.2 Sample collection for Diagnosis by Virus Isolation or Antigen Detection

Nasopharyngeal swabs should be collected as early as possible after the onset of fever to isolate or detect the virus (Timoney, 1996; van Maanen and Cullinane, 2002). A delay in veterinary intervention means that a low rate of isolation may result as peak shedding may be missed (Gildea et al., 2011a). Naïve horses may shed the EI virus for 7-10 days after infection. Partially immune horses only shed the virus transiently (van Maanen and Cullinane, 2002). On collection the nasopharyngeal swab must then be immersed in cool viral transport medium (VTM) and transported as quickly as possible to the laboratory. The sensitivity of a clinical test is generally defined as the ability of the test to correctly identify those patients with the disease. The sensitivity of these diagnostic tests depends on the stage of the disease at which

the swabs are collected. For instance, it was reported by Chambers et al. (1994) that when testing with Directigen Flu A, the strongest test reactivity was seen at two to three days after infection and this decreased thereafter.

### 2.6.3 Diagnostic Tests for Equine Influenza

**Table 2. 1 A Summary of the Diagnostic Tests Used to Detect Equine Influenza**

| Equine Influenza Diagnostic Methods evaluated in this study | | | |
|---|---|---|---|
| **Method** | **Test** | **Product Detected** | **Manufacturer/Reference** |
| **Virus Detection** | Inoculation of embryonated eggs followed by Hamagglutination (HA) test | Haemagglutinin antigen | OIE Manual, (2012) |
| **Viral Genome Detection** | Reverse Transcription-Polymerase Chain Reaction (RT-PCR) using both Light Cycler and Taqman technologies | Nucleic Acid | Roche, Mannheim, Germany Quinlivan et al. (2005) Foord et al. (2009) Lu et al.(2009) Kirkland et al. (2011) |
| **Viral Antigen Detection** | Directigen Flu A | NP | Becton-Dickinson and Co. (Maryland, USA) |
| | Espline Influenza A&B-N | NP | Fujirebio Inc. (Tokyo, Japan) |
| | ID Screen Influenza A Antigen Capture ELISA | NP | Innovative Diagnostics, Montpellier, France |
| **Antibody Detection** | Haemagglutination Inhibition (HI) | Antibody to Haemagglutinin | OIE Manual, (2012) |
| | Single Radial Haemolysis (SRH) | Antibody to Haemagglutinin | OIE Manual, (2012) |
| | ID Screen Influenza A Antibody Competition ELISA | Antibodies to nucleocapsid | Innovative Diagnostics, Montpellier, France |
| | ID Screen Influenza H7 Antibody Competition ELISA | Antibodies to H7 antigen | Innovative Diagnostics, Montpellier, France |

<u>Virus Isolation</u>

Equine Influenza virus can be isolated by inoculating 9 to 12 day old embryonated hen's eggs by the allantoic or amniotic routes. These eggs are incubated after infection at 34 ℃ and then refrigerated after 48 to 72 hours. If a negative result is

obtained following a haemagglutination (HA) test on the allantoic fluid, passaging is carried out, and may be carried out up to five times. Passaging involves infecting a new embryonated egg with the allantoic fluid harvested from the egg infected previously (OIE Manual, 2012). The haemagglutinating activity is checked after each passage (van Maanen and Cullinane, 2002). A HI test using specific antisera to H7N7 and H3N8 viruses can be carried out to confirm the subtype of influenza virus isolated (OIE Manual, 2012). Monolayer cultures of Madin Darby Canine Kidney (MDCK) cells maintained in serum-free medium with trypsin may also be used to isolate EI viruses (OIE Manual, 2012). Samples are infected onto a cell monolayer and inspected daily for evidence of cytopathic effect. A HA test is carried out on the supernatant fluids after a period of 7 days or earlier if cytopathic effect is observed. Negative supernatant fluids and those with titres <1/16 are repassaged up to five passages. Results with titres of $\geq$ 1:16 on the HA test are positive (OIE Manual, 2012).

Viral Genome Detection by Polymerase Chain Reaction

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) for viral diagnosis has become common practice in recent years. However, this method is more costly and is more technically demanding than many of the more traditional techniques. In the majority of veterinary diagnostic laboratories, RT-PCR has replaced VI as the front line test for the detection of EI. The samples tested are nasal secretions. It was reported by Quinlivan et al. (2005) that the real-time Light Cycler RT- PCR technology is more sensitive in the detection of positive samples than virus culture in embryonated eggs or the detection of the NP using the Directigen Flu A ELISA method. The methodology usually involves RNA isolation from nasopharyngeal swabs, reverse transcription and amplification of the resulting DNA using primers directed at a conserved region of the matrix gene (Fouchier et al., 2000; Quinlivan et al., 2005). A SYBR Green based RT-PCR assay detected positive samples up to day 7 after infection, one day more than any other diagnostic test (Quinlivan et al., 2004).

Viral Antigen Detection

The rapid antigen detection (RAD) kits used to detect the presence of the highly conserved NP of the influenza A and B virus provide results in a matter of minutes. EI virus is an Influenza A virus (van Maanen and Cullinane, 2002) and can therefore

be detected by these rapid kits (Yamanaka et al., 2008). Directigen Flu A manufactured by Becton–Dickinson and Co. (Maryland, USA) is capable of providing results in approximately 15 minutes, similarly to Espline Influenza A&B-N manufactured by Fujeribio Inc. (Tokyo, Japan). RAD kits could prove very useful in an outbreak in the absence of laboratory equipment or highly trained personnel (Yamanaka et al., 2008). Directigen Flu A and Espline Influenza A&B-N were reported by Yamanaka et al. (2008) to generate positive results in reasonable agreement with VI and RT-PCR on days 2 to 5 when most virus shedding occurs, but were less sensitive in early and late stages of infection. In their study, the Espline Influenza A&B-N and Directigen Flu A kits had almost equal sensitivities but the Directigen Flu A kit was less specific than the Espline Influenza A&B-N kit and gave false positive results in two of 93 nasal swabs.



**Figure 2. 1 Directigen Flu A test cassettes following incubation with cultured EI virus A/eq/Kildare/89 at 3 dilutions ($10^{-1}$, $10^{-2}$ and $10^{-3}$)**

The Directigen Flu A test kit shows the detection of antigen by a colour reaction, as can be seen in Figure 2.1. It works as an ELISA by binding influenza A antigen, to the enzyme conjugated monoclonal antibodies against

the NP on the membrane. The presence of a triangle, as seen above, indicates a positive result. The darker triangle indicates a more positive sample, arising from the presence of more antigen. The viral titers in Figure 2.1 were 55 HA units, 5.5 HA units and .55HA units (OIE Manual, 2008). The HA unit/titre is the last virus dilution which gives partial haemagglutination of chicken red blood cells (OIE Manual, 2012).
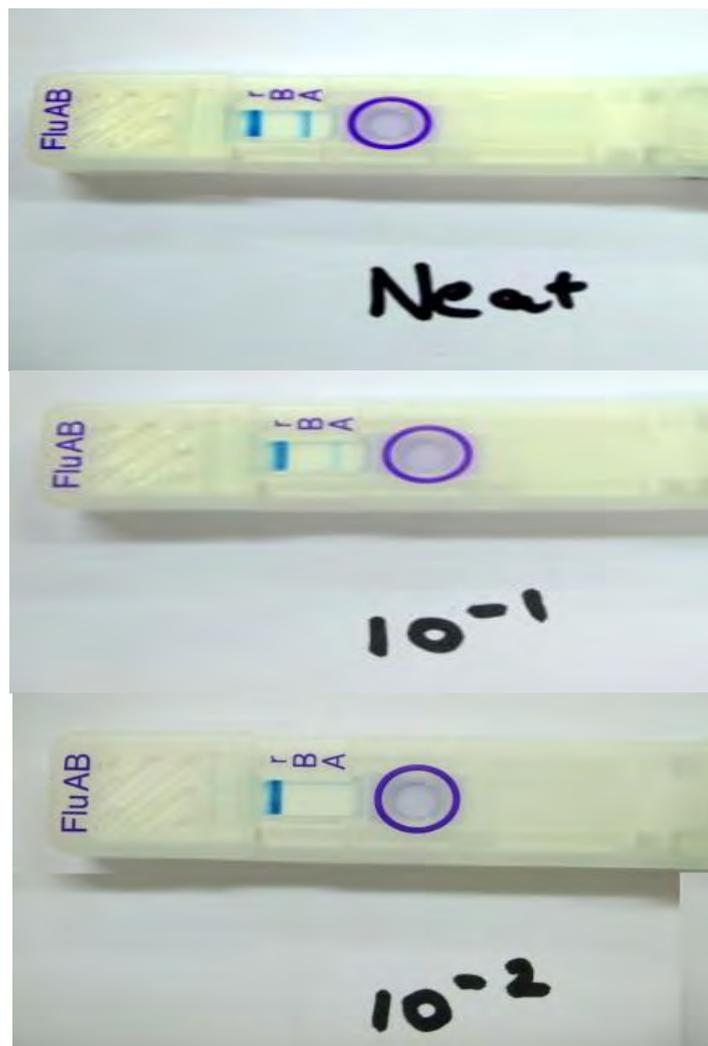


**Figure 2. 2 Espline Influenza A&B-N test cassettes following incubation with cultured EI virus A/eq/Kildare/89 at 3 dilutions ($10^{-1}$, $10^{-2}$ and $10^{-3}$)**

Espline Influenza A&B-N test kit shows the detection of antigen by the presence or absence of a line on the result window as can be seen in Figure 2.2. It uses immuno-chromatography technology based on the ELISA immunoassay principle. The presence of a line at the letter 'A' as seen above indicated a

positive result. This test detects the NP of influenza A. The darker line indicates a more positive sample, arising from the presence of more antigen. The viral titers at the three dilutions in Figure 2.2 were 550 HA units, 55 HA units and 5.5HA units (OIE Manual 2008).

ID Screen Influenza A Antigen Capture ELISA

The ID Vet ELISA kit, ID Screen Influenza A Antigen Capture (Innovative Diagnostics, Montpellier, France) is used for the detection of Influenza A virus NP in birds, horses and pigs by using wells coated with monoclonal antibody against the viral NP. According to the manufacturer's kit insert, the sensitivity of this ELISA was investigated by testing 10 strains of influenza A of different origins (avian, porcine, human) cultivated in eggs and 100% positivity was reported. The specificity of the ID Screen Influenza A Antigen Capture ELISA was confirmed by testing 100 cloacal samples and 50 liquid chicken samples that were known negatives. No false positive results were obtained. There have been no known published reports to date on the efficacy of this kit with equine influenza.

## 2.6.4 Comparison of Antigen Detection Tests

In a study of 125 swabs from horses and ponies carried out by Chambers et al. (1994), Directigen Flu A exhibited 83% sensitivity in comparison with virus isolation overall in this study. These results included virus detected in 26 out of 29 field cases and 13 out of 18 experimental samples. The Directigen Flu A kit was also able to detect subtype 1 EI H7N7. In the diagnosis of human influenza, Waner et al. (1991) found the sensitivity of Directigen Flu A compared to VI to be 100%. However, false positives were detected by Waner et al. (1991) when compared to cell culture; Yamanaka et al. (2008) also reported false positive results using this kit for EI, compared to RT-PCR. However, Yamanaka et al. (2008) also showed that neither of the two RAD kits, (Directigen Flu A or Espline Influenza A &B-N) was as sensitive as VI nor RT-PCR in the detection of EI virus from experimentally infected horses. In particular, these tests were seen to be less sensitive in early and late infection but produced results in reasonable agreement at the peak viral shedding days (2 to 5 days post infection). In the study by Yamanaka et al. (2008), Espline Influenza A&B-N

proved to be the better of the two RAD kits with superior specificity, but both Directigen Flu A and Espline Influenza A&B-N showed reasonable agreement in producing positive results. Quinlivan et al. (2004) found that the sensitivity of the Directigen Flu A for the detection of EI virus in nasal swabs from experimentally infected ponies was only 32% compared to that of VI using embryonated hen's eggs. In a follow up study using diagnostic samples for the detection of EI, Quinlivan et al. (2004) compared the sensitivity of RT-PCR, Directigen Flu A and VI. RT- PCR proved to be the most sensitive detection method with 20% of samples testing positive for EI compared to only 8% by Directigen Flu A and 5% by VI.

Overall, Directigen Flu A and Espline Influenza A&B-N are valuable tests as they are sensitive, rapid, and do not require laboratory expertise. These kits are not as sensitive as RT-PCR (Quinlivan et al., 2004), but they are more rapid and less labour intensive than either RT-PCR or VI. These RAD kits are not affected by freeze-thaw cycles (Yamanaka et al., 2008; Quinlivan et al., 2004). Directigen Flu A however has been reported as having poor specificity (Yamanaka et al., 2008). Espline Influenza A&B-N has been reported in some cases of human influenza as being less sensitive than Directigen Flu A (Hurt et al., 2007). ID Screen Influenza A Antigen Capture ELISA is capable of a mass screening of samples. It is a faster method of analysis than RT-PCR and VI, however, it is not as fast as Directigen Flu A or Espline Influenza A&B-N and it requires the use of laboratory equipment.

## 2.6.5 Antibody Detection

The most common tests used for quantifying antibodies against EI are the HI test and the SRH test (OIE Manual, 2012). A significant increase in antibody titre in paired sera (acute and convalescent samples) can be a useful indicator of recent infection. Since many horses have been vaccinated for EI or have been previously infected, the analysis of single samples is less informative and does not offer a definitive diagnosis. However, testing of single samples by SRH is useful to determine the immune status of a horse as a definite correlation between SRH antibody levels and protective immunity against EI. This has been established in both experimental challenge studies and in the field (Mumford et al., 1992; Mumford et al., 1994; Newton et al., 2000).

**2.6.6 Diagnostic tests for Equine Influenza Antibody Detection**

<u>Haemagglutination Inhibition</u>:

The HI test is widely used for the detection of antibodies to EI in serum samples after infection or vaccination. The titres are read as the highest dilution of serum that gives inhibition of agglutination of red blood cells by the virus HA (OIE Manual, 2012). The advantage of this test is its sensitivity; however, great care is needed to achieve reproducible results. Samples must be pre-treated to remove non-specific inhibitors and haemagglutinins before testing (Yamagishi et al., 1982). A seroconversion is defined as an increase of four-fold or greater in the titre between the acute and convalescent sample. The first sample should be taken at the onset of clinical signs and the second approximately two weeks later (OIE Manual, 2012).

<u>Single Radial Haemolysis test</u>

The SRH test is an immunodiffusion technique used for the detection of antibodies to influenza HA. This test uses influenza viral antigens coupled to the sheep red blood cells and guinea pig complement that have been suspended in agar plates. The test sera are added into the wells punched through the agar and the presence of antibodies is indicated by a clear haemolytic zone around the well (Morley et al., 1995a). This diagnostic technique combines the accuracy of single radial diffusion and the sensitivity of the HI test. A seroconversion is defined as a diameter increase of 50%, or 25mm$^2$ or greater between the acute sample at the onset of clinical signs and the convalescent sample collected approximately two weeks later (Mumford et al., 1995; Wood et al., 1994, Newton et al., 2000; OIE Manual, 2012).

<u>ID Screen Influenza A Antibody Competition ELISA</u>

This diagnostic ELISA kit from ID Screen Influenza A Antibody Competition (ID Vet, Montpellier, France) is used for the detection of antibodies to the internal nucleocapsid of the Influenza A virus in bird, pig and horse sera. This kit is therefore unlike the SRH and HI tests in that it is not subtype specific but is capable of detecting both Subtype 1 and Subtype 2 EI virus by detecting antibodies against the internal nucleocapsid of the Influenza A virus. The

manufacturer's sensitivity and specificity tests showed that chickens vaccinated with H7N1, or H5N9 and turkeys vaccinated with H5N9/H7N1, all demonstrated detectable antibodies. The specificity tests included 250 negative samples which were all detected as negative by the ID Screen Influenza A Antibody Competition ELISA.

ID Screen Influenza H7 Antibody Competition ELISA

This diagnostic kit is used for the detection of specific antibodies against the avian Haemagglutinin H7 of the Influenza A virus in bird serum. Subtype 1 EI H7N7 is no longer in circulation (Webster, 1993) but an H7N7 virus is contained in most EI vaccines. The antibodies to H7N7 in the equine sera may be detected using this ELISA method. There is no published record of this test being evaluated in horses previously. The sensitivity control was carried out by the manufacturers by testing serum from 20 chickens, 20 turkeys and 20 ducks which had HI titres of at least 1:16 against H7. These were all detected as positive by the ID Screen Influenza H7 Antibody Competition ELISA. The specificity control determined 250 disease-free birds as negative. These samples had previously tested negative for antibodies against the viral nucleopcapsid by the ID Screen Influenza A Antibody Competition ELISA.

## 2.6.7 Comparison of Influenza Antibody Detection techniques

The detection of antibodies against EI is traditionally quantified by SRH or HI tests. 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 more reproducible assay between laboratories (Mumford, 2000; Daly et al., 2007). The HI test provides results within hours. Russell et al. (1975) found in his study that there was good agreement between the HI test and SRH test for the detection of human antibodies to influenza virus strains. The SRH technique had an advantage in that it was unaffected by non-specific inhibitors and could distinguish between closely related strains of influenza.

The advantage of the ID Screen Influenza A Antibody Competition ELISA over the SRH and the HI tests is that it can detect seroconversions at an earlier stage (Read et al., 2012). This ELISA can also be used for its DIVA capacity to

manage and control the spread of EI in the event of an outbreak by recognising the vaccinated horses (Sergeant et al., 2009). The ID Screen Influenza H7 Antibody Competition ELISA is useful in monitoring vaccinal response to H7 viruses. Diagnosis can be carried out more rapidly with ELISA tests with 92 samples analysed per test run. However, despite its benefits, the ELISA is not ideal in all cases. Kittelberger et al. (2011) detected false positives with the ID Screen Influenza A Antibody Competition ELISA reporting a diagnostic specificity of 95.3%.

The SRH and HI test is a useful method of monitoring antibody levels in order to vaccinate strategically as they detect antibodies against the HA (Gildea et al., 2011c). This is in contrast to the ID Screen Influenza A Antibody Competition ELISA that detects antibodies to the nucleocapsid.

## 2.7 Equine Influenza Diagnosis: The Current Situation

Equine Influenza remains a serious and economically significant disease of horses. Vaccination and biosecurity provide strategies for the control of the disease in endemic areas. In the event of an influenza outbreak, rapid implementation of movement controls is important in limiting the spread of the disease. Prompt identification of the EI virus is key to effective control. Developments in molecular biology and ELISA technology have facilitated improvements in the rapidity and sensitivity of the diagnostic tests currently available. However, some of these new diagnostic tools have not been critically evaluated using clinical samples and have not been compared to the existing validated methodologies. That was the objective of the work described in this thesis. Comparative data on the efficacy of diagnostic tests could inform a clinical testing or screening programme. The objectives of the study were to compare the sensitivity of some of the newer diagnostic tests with the traditional VI and serological approaches using a variety of samples from acutely infected and convalescent animals, as well as animals vaccinated with a range of EI vaccine types.

# 3    *Materials and Methods*

## 3.1 Detection of Equine Influenza Virus

Three commercial kits used to detect the presence of influenza antigen were compared to the traditional 'gold standard' technique of VI in embryonated hen's eggs, and the technique now commonly used in many diagnostic laboratories, RT-PCR. Two of these commercial kits were RAD kits, Directigen Flu A manufactured by  Becton-Dickinson and Co. (Maryland, USA) and Espline Influenza A&B-N (Fujeribio Inc., Tokyo, Japan). The third detection method was an ELISA-based kit for antigen detection (ID Screen Influenza A Antigen Capture; ID Vet Innovative Diagnostics, Montpellier, France).

### 3.1.1 Sample Description

Nasopharyngeal swabs

All nasopharyngeal samples for this study were supplied by the Irish Equine Centre. The nasopharyngeal swabs were transported in VTM. The VTM consisted of phosphate-buffered saline with foetal calf serum (2% v/v), 100U of penicillin, 100μg of streptomycin and 5μg of amphotericin B (all from Gibco, Grand Island, NY, USA) per ml (Quinlivan et al., 2004). The samples were centrifuged at 1500 x g for 5 minutes and the supernatant was then tested, as described below.

### 3.1.1.1 Post Natural Infection Samples

Nasopharyngeal swabs were collected in 2007 and 2008 from 64 horses on eight premises where EI had been diagnosed by RT-PCR. Samples were collected from a polo yard (n=16), 3 racing yards (n=27), non Thoroughbred yard (n=6), showjumping yard (n=5), Thoroughbred stud (n=6) and a non Thoroughbred stud (n=4).  All samples were tested using the RAD kits, the ID Screen Influenza A Antigen Capture ELISA, VI and RT-PCR. RT-PCR, and in some cases VI, was carried out on the nasopharyngeal swabs following collection and prior to freezing. The samples were stored at -70°C following RT-PCR and VI and prior to testing using the two RAD kits and the ID Screen Influenza A

Antigen Capture ELISA in 2009. Previous results from this laboratory have shown that repeat freeze-thaw cycles can impact on the efficiency of VI but did not impact on RT- PCR or the Directigen Flu A test.

### 3.1.1.2 Post Challenge Samples

The samples from an experimental challenge study were made available for the evaluation of diagnostic tests. Nasopharyngeal swabs collected from seven non Thoroughbred weanling horses, which had been exposed to an aerosol of 10ml of A/eq/Kildare/89 at $10^6$ 50% egg infective doses ($EID_{50}$) (Mumford et al., 1990b), were used to compare the efficacy of virus detection methods. Samples were collected 1 day prior to challenge and each day for 14 consecutive days post challenge.

VI and RT-PCR were carried out on the nasopharyngeal swabs following collection and the samples were subsequently stored (-70ºC) prior to testing by Directigen Flu A, Espline Influenza A&B-N and the ID Screen Influenza A Antigen Capture ELISA. The Directigen Flu A and Espline Influenza A&B-N positive results were graded 1 to 3, with 3 being the strongest.

### 3.1.1.3 Comparison of Sensitivity of Commercial Kits

The sensitivities of the three kits used to test for EI virus were compared using serial ten-fold dilutions of A/eq /Kildare/89 grown to a HA concentration of 550 in embryonated hen's eggs at the Irish Equine Centre. The HA unit/titre is the last virus dilution which gives partial haemagglutination of 1 % chicken red blood cells (Powell, 1991; OIE Manual, 2012). The lowest dilution of virus detected by each kit was recorded as the HA unit/titre. The dilutions tested were 0, $10^{-1}$, $10^{-2}$ and $10^{-3}$, corresponding to 550, 55, 5.5 and 0.55 HA units, respectively.

## 3.2 Tests Procedures

### 3.2.1 Directigen Flu A

The Directigen Flu A, an *in vitro* enzyme immunoassay membrane test manufactured by Becton-Dickinson and Co. (Maryland, USA), was used in accordance with the manufacturer's instructions. However, the samples were centrifuged and frozen prior to analysis. The manufacturer has noted that the sensitivity of the test may decrease with repeated freeze-thaw cycles. Chambers et al. (1994) have used both fresh and frozen samples in the evaluation of the Directigen Flu A test and noted an apparent increase in specificity using frozen samples. Extracted nasopharyngeal samples (0.125ml) were added to the test device. Influenza A antigen, which bound to the membrane surface, was detected by the addition of enzyme conjugated monoclonal antibodies against the NP and two substrates (0.4mM chromogen, with 0.2% sodium azide and 0.78mM chromogen, with 0.2% sodium azide). Each Directigen Flu A test device has a H1N1 antigen spot in the centre of the membrane, which develops as a purple dot indicating the integrity of the test. The development of a purple triangle surrounding the dot is indicative of a positive reaction. A positive result was indicated by the presence of a purple triangle after 5 minutes incubation at room temperature. The intensity of the triangle was graded from 1 to 3 as described by Quinlivan et al. (2004), with 3 being a dark purple triangle, 2 a light coloured triangle and 1 an outline of a triangle.

### 3.2.2 Espline Influenza A&B-N

All samples evaluated using this test had been frozen, as described by Yamanaka et al. (2008). The Espline Influenza A&B-N kit is an immuno-chromatography cassette-style test which uses anti-influenza type A and B virus monoclonal antibodies. Each nasopharyngeal swab was soaked in the extraction solution provided in the kit. Two drops of the sample, diluted in the extraction fluid (approx. 30μl), were dropped onto the sample window which contains alkaline phosphatase labelled monoclonal antibody against the NP. Antigen antibody complexes migrated to fixed antibody where a positive sample was indicated by the production of a blue line on addition of substrate. In this study, the intensity of the line was graded from 1 to 3, with 3 being a strong positive.

### 3.2.3 ID Screen Influenza A Antigen Capture

All samples evaluated using this test had been frozen. The manufacturer's instructions do not stipulate a requirement for fresh samples. This ELISA which detects the NP of influenza viruses of birds, horses and pigs, was carried out in accordance with the manufacturer's instructions. Briefly, the wells of the test plate were coated with anti-Antigen A (NP) monoclonal antibody. The nasopharyngeal samples were diluted 1:2 in dilution buffer, added to the test wells and incubated at 37°C for an hour. Bound antigen was detected with peroxidase labelled antibody. .

The test is valid according to the manufacturer Innovative Diagnostics (Montpellier, France), if the mean of the positive control OD (ODPC) is greater than 0.350 and the ratio of the mean values of the positive and negative controls (ODPC and ODNC) is greater than 3. The optical density (OD) value was measured using Magellan Software and the Sunrise Absorbance Reader (450nm filter) manufactured by Tecan (Männedorf, Switzerland). The cut-off was set at 4 times the mean OD value of the negative controls. Samples with an OD value less than or equal to the cut-off were considered negative, and samples with an OD value greater than the cut-off were considered positive.

### 3.2.4 Virus Isolation

Virus isolation was performed using both fresh and frozen samples. Previous work from this laboratory has developed a protocol, involving the inclusion of 2% foetal calf serum in the VTM as a cryoprotectant, that reduces the impact of freeze-thaw cycles on the success of VI (Quinlivan 2004). Embryonated hen's eggs (9-12 day old) were used to isolate the EI virus from the nasopharyngeal swabs. A sterile forceps was used to squeeze the nasal secretions from the swab prior to its removal from the VTM. A 100μl aliquot of each sample was then inoculated into the allantoic cavity of three embryonated eggs. The eggs were incubated at 34°C for two to three days before being refrigerated at 4°C overnight. The allantoic fluid was then harvested and tested for the presence of influenza antigen by carrying out a HA test using 1% chicken erythrocytes (OIE Manual, 2008).

Reference virus strains, EHV-1, EHV-2 and EHV-4 and Rhinovirus 1 and 2 of known Tissue Culture Infectious Dose 50 (TCID$_{50}$) were supplied by the

serology laboratory in the Irish Equine Centre. Both $TCID_{50}$ and Egg Infectious Dose 50 ($EID_{50}$) were calculated using the Karber formula (Karber, 1931).

### 3.2.5 Reverse Transcription-Polymerase Chain Reaction

Reverse Transcription-Polymerase Chain Reaction was performed primarily using fresh samples. This laboratory has previously demonstrated a minimal impact of freeze-thaw on the sensitivity of PCR (Quinlivan et al., 2004). The matrix primers designed by Fouchier et al. (2000) were used in RT-PCR to test the samples from the challenge study. Real-time RT-PCR was carried out using the Light Cycler RNA Amplification Kit SYBR Green 1 (Roche, Mannheim, Germany). RNA was extracted firstly from 140μl of the nasopharyngeal swabs using the Qiamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Following this a 10μl RT-PCR mix was prepared, consisting of 2.0μl of 5x reaction mix, 6.0mM $MgCl_2$ , 0.2μl reverse transcriptase enzyme mix, supplied in the kit and 1.5μl of RNA. Primers were at a concentration of 0.5μM. Reverse transcription was carried out at 55°C. This was then followed by an initial denaturation step at 95°C for 30 seconds. cDNA was amplified in 40 to 50 cycles at 95°C and held for 0 seconds at 65°C for 5 seconds and 72°C for 10 seconds with fluorescence data acquired at the end of each cycle in a single step. Amplification was stopped and a standard melting curve analysis was performed once the plateau phase of the PCR had been reached. The melting curve consisted of 95°C held for 0 seconds, 65°C for 10 seconds and a 0.1°C second rise to 95°C with continual fluorescence measurement (Quinlivan et al., 2005).

The clinical samples were tested by real-time RT-PCR carried out on the ABI Taqman 7500 platform using the AgPath-ID one-step RT-PCR kit (Ambion/Applied Biosystems, Foster City, CA, USA). Primer probe sets against a highly conserved sequence in the matrix gene were used (Foord et al., 2009). PCR conditions employed were as follows: 45° C for 10 minutes, 95 ° C for 10 minutes, 45 cycles of 95 ° C for 15 seconds and 60 ° C for one minute.

## 3.3 Detection of antibodies to Equine Influenza Virus

The detection of antibodies was investigated using the ID Screen Influenza A Antibody Competition ELISA manufactured by Innovative Diagnostics (Montpellier, France). The results from these tests were compared with the results from SRH assays, and, in clinical cases post natural infection, also with the results from HI assays. Seronegative horses were defined in this study as horses that had no antibodies to the virus. Seropositive horses were defined as horses that had antibodies to the virus.

### 3.3.1 Sample Description

#### Serum Samples

All serum samples used were provided by the Irish Equine Centre. Each blood sample was initially prepared by centrifugation at 2200 x g for 10 minutes to separate the red blood cells from the serum. The serum was then removed and the samples aliquoted and stored at -20°C.

### 3.3.1.1 Horses Post Natural Infection

Paired sera samples (n=203) were collected between 2 and 291 (mean 19.4 days SE $\pm$ 3.81) days apart from horses during EI outbreaks in 14 premises between 2007 and 2010. The seronegative horses of known age ranged from <6 months to 10 years with a mean of 3.7, SE$\pm$ 0.76 years. The seropositive horses of known age ranged from >1 yr to 25+ years with a mean of 5.6, SE$\pm$ 0.30 years. The serum was collected from seven horses from non-Thoroughbred yards, 27 from showjumping yards, 103 from racing yards, five from hunting yards, 19 from riding schools, 28 from Thoroughbred studs, two from Thoroughbred farms and 12 from a polo yard. These samples were tested by the ID Screen Influenza A Antibody Competition ELISA, SRH assay and the HI test. The ID Screen Influenza A Antibody Competition ELISA kit was used to determine if seroconversions could be detected in the case of a natural outbreak by determining if there was a decrease of 50% or greater in the competition percentage between the acute and convalescent samples.

### 3.3.1.2 Seronegative Horses Post Challenge

Sera from 14 horses that participated in experimental challenge studies were made available for the evaluation of serology tests. Samples were taken from non-Thoroughbred weanlings which were challenged with A/eq/Kildare/89 at $10^6$ EID$_{50}$, a dose demonstrated by Mumford et al. (1990b) to produce clinical signs. Samples were taken at 1 day prior to challenge, 7 and 14 days post challenge. These samples were tested by SRH and ID Screen Influenza A Antibody Competition ELISA.

### 3.3.1.3 Seronegative Horses Post Vaccination

Sera collected and tested by SRH for a comparative vaccine study (Gildea et al., 2011c) were tested with the ID Vet ID Screen Influenza A Antibody Competition ELISA. Sixty Thoroughbred weanlings (circa 6-10 months of age) which tested seronegative using the SRH test received their primary vaccination course in line with regulations of the Irish Turf Club (Turf Club Rule Book, 2011). The vaccines that these animals received included: a whole vaccine Duvaxyn IE T Plus (Fort Dodge; 11 horses) a whole vaccine Equilis Resequin (Intervet; 11 horses), an inactivated subunit vaccine Equip FT (Pfizer; 14 horses), inactivated subunit vaccine Equilis Prequenza Te (Intervet; 13 horses) and a canary pox recombinant vaccine Proteq Flu Te (Merial; 11 horses). The vaccination and sampling regime have been described previously (Gildea et al., 2011c). Briefly, blood samples were collected on the day of first vaccination, (V1) and on V1+2 weeks. The weanlings received a second vaccination (V2) 5 weeks after V1, and were sampled on the day of second vaccination (V2), V2+2weeks, V2+13 weeks. The weanlings received a third vaccination (V3) 26 weeks after V2, and were sampled on V3, V3+2 weeks, V3+13 weeks, V3+ 26 weeks. The samples from these weanlings were used in this study to determine the DIVA capacity of the ID Screen Influenza A Antibody Competition ELISA in horses vaccinated with the canary pox recombinant and other subunit vaccines and to determine the sensitivity of the test to detect antibodies in vaccinated horses at different stages of their vaccination programme.

### 3.3.1.4 Seropositive Horses Post Vaccination

Sera collected and tested by SRH in a comparative vaccine study concerning annual booster vaccination of National Hunt horses (Gildea et al., 2011b) were tested by the ID Screen Influenza A Antibody Competition ELISA. Forty four seropositive horses (as determined by SRH prior to vaccination) were vaccinated with whole virus vaccines Duvaxyn IE T Plus (7), Prevac T Pro (8), Equilis Resequin (7), subunit vaccines, Equilis Equenza T (7), Equip FT (9) and the canary pox recombinant vaccine Proteq Flu Te (6). The acute samples were taken on the day of vaccination and the convalescents were taken at two weeks, 4 weeks, 12 weeks and 24 weeks post vaccination.

### 3.3.1.5 H7N7 Samples Post Natural Infection

Paired sera from 31 horses which were naturally infected in 2009 or 2010 with subtype 2 EI (H3N8) were tested by SRH, HI and the ID Screen Influenza H7 Antibody Competition ELISA. All of these horses seroconverted to H3N8 virus by SRH. The acute samples included both seronegative and seropositive samples for subtype 1 (H7N7) EI.

### 3.3.1.6 H7N7 Samples Post Vaccination

The serum samples from the comparative vaccine study in weanlings (Gildea et al., 2011c) were tested with the ID Screen Influenza H7 Antibody Competition ELISA to evaluate the ability of the test to detect antibodies against H7N7 at different stages of a primary vaccination programme. The ELISA results were compared with SRH results. The vaccines that these animals received included: a whole vaccine Duvaxyn IE T Plus (Fort Dodge; 12 horses) a whole vaccine Equilis Resequin (Intervet; 12 horses), an inactivated subunit vaccine Equip FT (Pfizer; 14 horses), inactivated subunit vaccine Equilis Prequenza Te (Intervet; 14 horses) and a canary pox recombinant vaccine Proteq Flu Te (Merial; 11 horses).

## 3.4 Test Procedures for the Detection of Antibodies

### 3.4.1 Haemagglutination Inhibition Test

The HI test was used in this study to detect the presence of antibodies to EI Haemagglutinin. The serum samples (150µl) used in the HI test were inactivated to eliminate any non-specific activity. Inactivation was performed by the addition of 300µl of a .016M solution of potassium periodate (BDH Anala R, Poole, England), as described by OIE Manual, 2008 to remove nonspecific haemagglutinins. The sample was then incubated at room temperature for 15 minutes. Glycerol (150µl of a 3% solution) was added also to the sample to neutralise any excess periodate solution and the sample was incubated for 15 minutes (room temperature) before being incubated at 56°C water bath for 30 minutes.

The inactivated serum (diluted 1:4) by the addition of potassium periodate and glycerol) was then diluted in a 96 well V-bottom plate (Sterilin, Caerphily, UK) to a 1:8 dilution with PBS and was titred by making serial double dilutions across the plate. Twenty five µl of antigen at 4 HA units was added to all of the test wells of the plate. The antigens used in this study were A/eq/Prague/56, A/eq/Kildare/89 and A/eq/Kildare/92 or A/eq/Meath/07. A HA control was set up for each antigen to ensure that they were correctly diluted. The test plate was then left to incubate for 30 minutes at room temperature. Fifty µl of 1% chicken erythrocytes in PBS were added to the test wells. The test plates were incubated at room temperature for a further 45 minutes. The plate was then read and the end point of HA was determined. The HI titre of each serum sample was the highest dilution of serum that caused 50% inhibition of HA of red blood cells (OIE Manual, 2008). A titre of 1:16 or greater was considered a positive result. A seroconversion was defined as an increase of four-fold or greater in the titre between paired samples.

### 3.4.2 Single Radial Haemolysis Test

This test was used to determine antibody levels in serum to EI Haemagglutinin. The antibodies in the sera are allowed to diffuse through the agar to cause haemolysis of virus-treated erythrocytes in the presence of complement.

Serum samples and both the negative and positive controls were inactivated by incubation at 56°C for 30 minutes to eliminate any non-specific activity. Blood agar plates were made up in tissue culture dishes (Sarstedt, Nümbrecht, Germany) using 1% agarose (Sigma-Aldrich, Missouri, USA). These contained 8% sheep red blood cells, optimized viral antigen coupled to chromium chloride and guinea-pig complement (0.5ml of guinea-pig complement in 13ml of agarose combined with 1.5ml of sensitised sheep red blood cells, Harlan Seralab, Leicestshire, England) (OIE Manual, 2008). The antigens used included A/eq/Prague/56 (H7N7), and A/eq/South Africa/03 (H3N8), A/eq/Newmarket/2/93 (H3N8), A/eq/Meath/07 (H3N8) and A/eq/Kildare/92 (H3N8). A/eq/South Africa/4/03 (H3N8) was used for the comparison of SRH antibody responses with ELISA responses post booster vaccination. Control antisera against A/eq/Newmarket/77 (H7N7), A/eq/Newmarket/2/93 (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.

Three mm wells were punched in the agar plates and 10µl of the serum sample was added. Plates were incubated at 34°C for 20 – 24 hours and the results were read. The lysis zone was measured with a viewer and digital recording apparatus (Mitutoya, Aurora, Illinois, USA) in 2 diameters. A clear, haemolytic zone around a well was considered a positive result. The following formula was then used to calculate the area of the zone of haemolysis (OIE Manual, 2008):

The zone of lysis = Area (mm$^2$) of the zone

$$\prod r^2 = 3.14 \times \left( \frac{\text{radius 1} + \text{radius 2}}{2} \right)^2$$

A diameter increase of 50% or 25mm$^2$ between paired samples was defined as a seroconversion (Newton et al., 2000; OIE Manual, 2008). A poor responder to vaccination was defined as a failure to mount a mean H3N8 antibody response of $\geq$ 25mm$^2$.

### 3.4.3 ID Vet: ID Screen Influenza A Antibody Competition

This ELISA kit developed by ID Vet (Innovative Diagnostics, Montpellier, France) was used to detect antibodies in serum against the internal nucleocapsid of the Influenza A virus. The wells of the test plate provided were coated with Antigen A. The serum samples and kit controls were diluted to a 1:10 dilution in dilution buffer provided with the kit. Next, 100μl aliquot of positive controls, negative controls or serum samples were added to the plate. If antibodies to antigen A were present in the serum, an antibody-antigen complex would form. The plate was incubated for 1 hour at 37°C. The plate was then washed 5 times using the wash solution provided. Next, 50μl of anti-antigen A-peroxidase conjugate was then added to the test plate and allowed to incubate for 30 minutes at room temperature. This conjugate forms an antigen-conjugate-peroxidase complex when it fixes to any remaining free antigen A epitopes. The plate was then washed 3 times to eliminate excess conjugate. Fifty μl of the substrate TMB was then added and incubated for 10 minutes at room temperature. Stop solution (0.5M $H_2SO_4$; 50μl) was then added to all the test wells to stop the reaction. The OD value of each well was read at 450nm using Magellan software and the Sunrise Absorbance Reader (Tecan; Männedorf, Switzerland).

The competition percentage was calculated for each sample tested. The test is valid according to the manufacturer; Innovative Diagnostics (Montpellier, France), if the mean value of the negative control OD is greater than 0.700 and the mean value of the positive control is less than 30% of the OD of the negative control. The competition % is calculated for each sample as follows:

**Competition % =** $\dfrac{\textbf{OD}_{\textbf{SPECIMEN}}}{\textbf{OD}_{\textbf{NC}}}$

The manufacturer's suggested interpretation of the results is as follows:

Competition % $\leq$ 45 %           =           Positive

45% < Competition% < 50 %   =           Doubtful

Competition % $\geq$ 50 %         =           Negative

A sample that produced a doubtful result was classified as negative. A seroconversion was defined as a decrease in competition percentage of 50% or more, or a change from seronegative to seropositive between acute and convalescent samples. A poor responder to vaccination was defined as a failure to convert from negative to positive or a response that resulted in less than a 50% decrease in competition percentage.

### 3.4.4 ID Vet: ID Screen Influenza H7 Antibody Competition

This ELISA kit was used for the detection of specific antibodies against the H7 Antigen of the Influenza virus. The wells of the test plate were coated with the H7 antigen. One hundred μl of samples or kit controls were added to the microwells at a dilution of 1:10 as appropriate and the plate was incubated at 37°C for 1 hour. An antibody-antigen complex was formed if anti-H7 antibodies were present in the serum and this complex then masks the H7 epitopes. The wells were washed 3 times and 50μl of conjugated was added. The plate was subsequently incubated at room temperature for 30 minutes. The wells were washed 3 times before 50μl of substrate solution was added and incubated for 10 minutes at room temperature followed by 50μl of stop solution (0.5M $H_2SO_4$). The resulting colour developed depends on the quantity of specific antibodies present in the specimen. The plate was read at 450nm using Magellan software and the Sunrise Absorbance Reader (Tecan; Männedorf, Switzerland). In the absence of antibodies, a colour is seen whereas, in the presence of antibodies, no colour is seen. The test is valid according to the manufacturer Innovative Diagnostics (Montpellier, France) if the mean of the negative control OD is greater than 0.700 and the mean value of the positive control is less than 30% of the OD of the negative control. The competition % is calculated for each sample as follows:

$$\text{Competition \%} = \frac{OD_{SPECIMEN}}{OD_{NC}}$$

The suggested interpretation of the results is as follows:

| | | |
|---|---|---|
| Competition % ≤ 50 % | = | Positive |
| 50% < Competition% < 60 % | = | Doubtful |
| Competition % ≥ 60 % | = | Negative |

A seroconversion was described as a decrease in competition % of 50% or more. A sample with a doubtful (competition percentage greater than 50% and less than 60%) result was described as a negative result.

## 3.5 Statistical Analysis

The inter-rater agreement between diagnostic tests was calculated using Cohen's kappa test (Cohen, 1960). The diagnostic tests were compared with each other under different circumstances of infection. Calculations were carried out using GraphPad software www.graphpad.com/quickcalcs/index.cfm and Vassarstats, http://vassarstats.net/kappa.html. Following the calculation, the results indicated the level of agreement between tests based on the principle by Landis and Koch (1977); Poor = $\leq 0$, Slight=0.01-0.2, Fair = $0.21 - 0.4$, Moderate = $0.41 - 0.6$, Substantial = $0.61 - 0.8$, and Almost perfect = $0.8 - 1.00$. The number of agreed positives and negatives along with the number of disagreements were calculated to determine the kappa scores in each population. Doubtful results as defined by ELISA tests were described as negative in each calculation. Correlation coefficient analysis and one-way ANOVA analysis was carried out using Microsoft Excel 2007. For the comparison of vaccines a one-way ANOVA was used with Mann-Whitney and a Bonferroni correction being applied so that all effects are reported at 0.005 significance level.

# *4    Results*

## 4.1 Detection of Equine Influenza Virus

### 4.1.1 Detection of Equine Influenza in Naturally Infected Horses

Seventy five samples collected from 64 horses on eight premises were tested for EI by the following tests:

(1) Directigen Flu A, RAD

(2) Espline Influenza A&B-N, RAD

(3) ID Screen Influenza A Antigen Capture ELISA

(4) VI

(5) RT-PCR

The samples were collected from horses in a variety of premises where natural outbreaks of EI were recorded. The percentage of positives detected by each test is summarised in Figure 4.1.
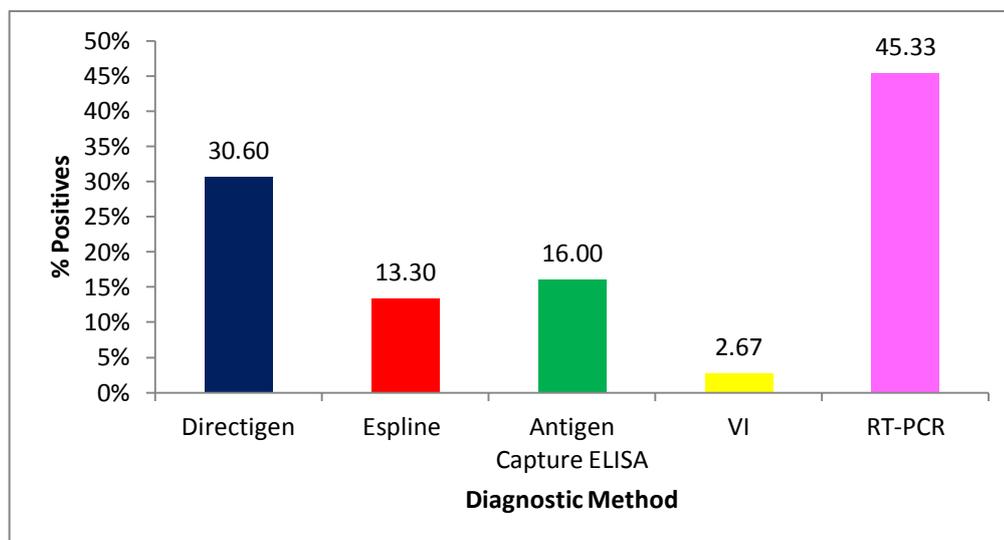


**Figure 4. 1 Comparison of EI detection methods in naturally infected horses (n=75)**

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

RT-PCR detected the highest number of positive samples (45.33%) and therefore was the most sensitive test in this study followed by Directigen Flu A (30.60%), ID Screen Influenza A Antigen Capture ELISA (16.00%), Espline

Influenza A&B-N (13.30%) and VI (2.67%) (Figure 4.1). Directigen Flu A was the most sensitive RAD kit followed by ID Screen Influenza A Antigen Capture ELISA and Espline Influenza A&B-N. The positive samples detected by ID Screen Influenza A Antigen Capture ELISA and Espline Influenza A&B-N were all detected by Directigen Flu A. Furthermore, all Espline Influenza A&B-N positives and 9 of 12 ID Screen Influenza A Antigen Capture ELISA positives were detected as strong positives (3) by Directigen Flu A (Table 4.1). RT-PCR negative samples tested negative by all other assays. Thirty four of the 75 samples were detected as positive by one or more method. Seven swabs were positive by RT-PCR and the three commercial kits but no virus was subsequently isolated. Only one horse, horse 49 was detected as a positive by all five methods (Table 4.1). This horse was the index case on the training yard of 56 horses. At the time of sampling this horse was exhibiting clinical signs and had not been vaccinated in over 15 months. Horse 50 was the second horse on this yard to exhibit clinical signs, three days after the index case. Nasopharyngeal swab samples were taken from both horses on the same day, however, horse 50 was detected as positive by Directigen Flu A, RT-PCR and VI, but not by Espline Influenza A&B-N or ID Screen Influenza A Antigen Capture ELISA. Twelve positive samples detected by RT-PCR were from subclinically infected horses. Five of these were detected by Directigen Flu A, two by ID Screen Influenza A Antigen Capture ELISA and only one was detected by Espline Influenza A&B-N. Nine samples detected by RT-PCR were from vaccinated horses. Seven of these were detected by Directigen Flu A, five by ID Screen Influenza A Antigen Capture ELISA and only one was detected by Espline Influenza A&B-N.

**Table 4. 1 Detection of EI in nasopharyngeal swabs from naturally infected horses**

| Horse | Clinical Signs | Vaccination | Directigen | Espline | Antigen Capture ELISA | VI | RT-CR | Serology SC | Serological status of acute sample |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Yes | No | - | - | - | - | + | No | H3N8 titre |
| 5 | Yes | No | +(3) | +(1) | - | - | + | Yes | H3N8 titre |
| 6 | Yes | No | +(1) | - | - | - | + | Yes | H3N8 titre |
| 8 | Yes | No | +(3) | +(1) | - | - | + | Yes | No Ab |
| 10 | No | No | +(1) | - | - | - | + | No | No Ab |
| 11 | No | No | +(3) | +(1) | + | - | + | N/A | H3N8 titre |
| 12 | No | No | +(1) | - | - | - | + | Yes | H3N8 titre |
| 17 | Yes | No | - | - | - | - | + | No | No Ab |
| 21 | Yes | 1 Dose | +(3) | - | + | - | + | No | No Ab |
| 22 | Yes | No | +(1) | - | - | - | + | Yes | H3N8 titre |
| 23 | Yes | Yes | +(3) | +(2) | + | - | + | Yes | H3N8 titre |
| 24 | Yes | Yes | +(2) | - | + | - | + | Yes | H3N8 titre |
| 25 | Yes | Yes | +(1) | - | - | - | + | No | No Ab |
| 26 | Yes | Yes | +(2) | - | + | - | + | Yes | H3N8 titre |
| 27 | Yes | No | +(1) | - | - | - | + | Yes | H3N8 titre |
| 28 | Yes | No | +(1) | - | - | - | + | Yes | H3N8 titre |
| 34 | No | Out of Date | +(1) | - | - | - | + | Yes | H3N8 titre |
| 40 | Yes | Unknown | - | - | - | - | + | N/A | No Ab |
| 41 | No | Unknown | - | - | - | - | + | N/A | No Ab |
| 43 | No | Unknown | - | - | - | - | + | N/A | No Ab |
| 45 | Yes | Unknown | +(3) | +(2) | + | - | + | Yes | H3N8 titre |
| 46 | No | Unknown | - | - | - | - | + | N/A | No Ab |
| 48 | Yes | Unknown | +(3) | +(1) | + | - | + | Yes | H3N8 titre |
| 49 | Yes | Out of Date | +(3) | +(1) | + | + | + | Yes | H3N8 titre |
| 50 | Yes | Yes | +(2) | - | - | + | + | Yes | H3N8 titre |
| 54 | Yes | No | - | - | - | - | + | No | No Ab |
| 57 | No | Yes | +(2) | - | + | - | + | No | No Ab |
| 61 | Yes | Unknown | +(3) | +(2) | + | - | + | Yes | H3N8 titre |
| 63 | Yes | Unknown | +(3) | +(2) | + | - | + | N/A | No Ab |
| 64 | Yes | Unknown | +(3) | +(2) | + | - | + | N/A | No Ab |
| 67 | No | Yes | - | - | - | - | + | No | No Ab |
| 68 | No | Unknown | - | - | - | - | + | No | No Ab |
| 69 | No | Out of Date | - | - | - | - | + | No | No Ab |
| 70 | No | Yes | - | - | - | - | + | No | No Ab |

**Clinical Signs=Clinical signs observed during the outbreak, SC = Seroconversion to H3N8 by HI test (SC: Yes =Seroconversion, SC: No = No seroconversion, SC:N/A = One sample only) H3N8 titre= High antibody titre to H3N8 EI but no detectable antibody titre to H7N7 EI, No Ab= No evidence of antibodies from natural infection in the acute sample**

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

## 4.1.2 Detection of Equine Influenza in Post Challenge Samples

The results of the detection of EI in nasopharyngeal swabs from experimentally infected foals by VI, RT-PCR and the three commercial kits are summarised in Figure 4.2 and Figure 4.3. RT-PCR detected the highest number of positive samples (49.04%) and therefore was the most sensitive test in this study followed by VI (35.58%), Directigen Flu A (16.35%), Espline Influenza A&B-N (2.88%) and ID Screen Influenza A Antigen Capture ELISA (0.96%).
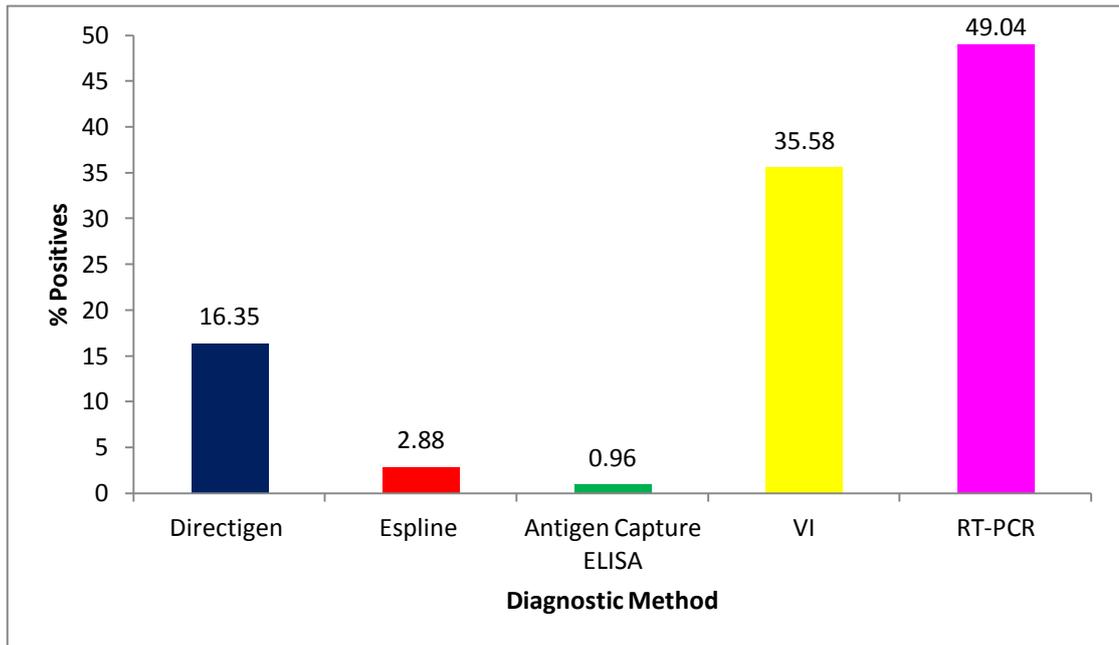


**Figure 4. 2 Comparison of EI detection methods in nasopharyngeal swabs (n=104) from experimentally challenged foals**

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**
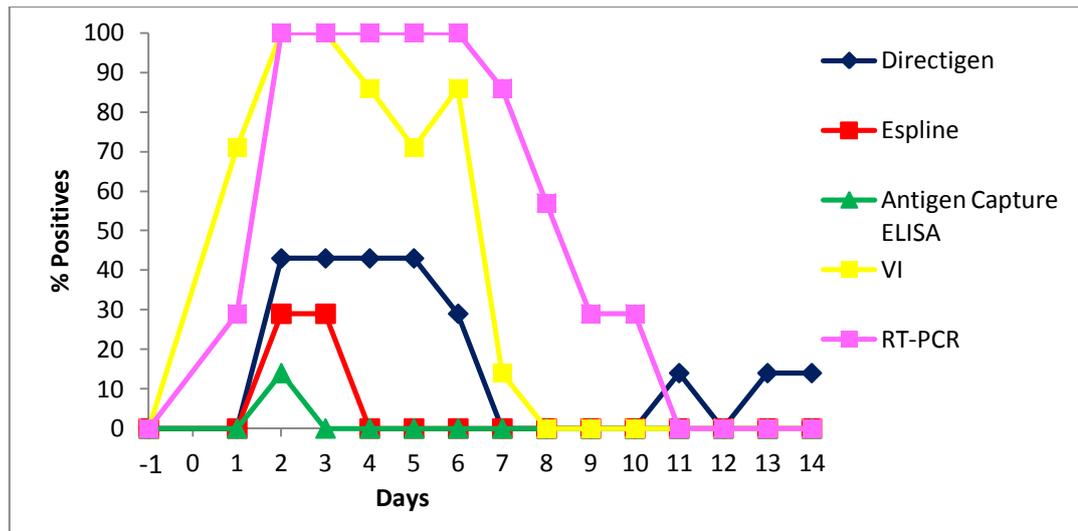
**Figure 4. 3 Detection of EI in nasopharyngeal swabs (n=104) collected from foals on Day -1 to Day 14 post challenge**

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

Peak viral shedding occurred from Day 2 to Day 6 post challenge. Virus was isolated from all (100%) of the samples collected on Day 2 and Day 3, 86% of the samples collected on Day 4 and 6 and 71% of the samples collected on Day 5 (Table 4.2). The VI results correlated with the results of RT-PCR but VI lacked the sensitivity of RT-PCR in the later stages. On Day 1, 3 of the VI positive samples were not detected by RT-PCR. Overall however, RT-PCR was the most sensitive method of detection. Positives were detected from Day 1 to Day 10 with the majority of samples being identified as positive at the time of peak shedding, Day 2 to Day 6. A gradual decrease in the number of positive samples detected by RT-PCR was observed after Day 6. None of the antigen detection kits used was as sensitive as either VI or RT-PCR post challenge.

**Table 4. 2 $EID_{50}$ of EI in nasopharyngeal swabs collected from Day 1 to Day 7 post challenge**

| Day | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| No. of Positives | 5/7 | 7/7 | 7/7 | 6/7 | 5/7 | 6/7 | 1/7 |
| Mean $EID_{50}$/ml | $10^{0.90}$ | $10^{2.39}$ | $10^{2.32}$ | $10^{2.25}$ | $10^{3.00}$ | $10^{1.88}$ | $10^{1.5}$ |
| Standard Error | $10^{0.187}$ | $10^{0.349}$ | $10^{0.223}$ | $10^{0.342}$ | $10^{0.468}$ | $10^{0.301}$ | n/a |

**Table 4. 3 $EID_{50}$ of EI in nasopharyngeal swabs positive by RAD kits**

| Horse | Day | Directigen | Espline | Antigen Capture ELISA | Titre/ml |
|---|---|---|---|---|---|
| | | | | | |
| B | | Pos 1 | Neg | Neg | $10^{2.5}$ |
| F | 2 | Pos 3 | Pos 2 | Pos | $10^{3.25}$ |
| G | | Pos 2 | Pos 1 | Neg | $10^{4}$ |
| D | 3 | Pos 1 | Neg | Neg | $10^{1.75}$ |
| E | | Pos 1 | Pos 1 | Neg | $10^{3.5}$ |
| F | | Pos 1 | Neg | Neg | $10^{2.25}$ |
| B | 4 | Pos 1 | Neg | Neg | $10^{1.5}$ |
| E | | Pos 2 | Neg | Neg | $10^{3.5}$ |
| F | | Pos 1 | Neg | Neg | $10^{2.5}$ |
| A | 5 | Pos 2 | Neg | Neg | $10^{4.5}$ |
| E | | Pos 1 | Neg | Neg | $10^{1.75}$ |
| C | | Pos 1 | Neg | Neg | $10^{1.75}$ |
| D | 6 | Pos 1 | Neg | Neg | $10^{1.75}$ |

Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction
The positives are graded from 1 to 3, with 3 being the strongest and 1 the weakest

Directigen Flu A was the most sensitive of the commercial RAD kits (Figure 4.2). The $EID_{50}$ of the Directigen Flu A positive samples ranged from $10^{1.5}$ to $10^{4.5}$ (Table 4.3). Positives samples were detected by Directigen Flu A on Days 2 to 6 when virus shedding peaked. A grade 3 positive was detected on Day 2 while grade 2 positives were detected on Days 2, 4 and 5. All other samples detected by Directigen Flu A were grade 1 positives. This kit also detected weak positives (grade 1) on Days 11, 13 and 14 but no virus was isolated. Only three

positive samples were detected by the Espline Influenza A&B-N RAD kit. The $EID_{50}$ of the Espline Influenza A&B-N positive samples ranged from $10^{3.5}$ to $10^4$ (Table 4.3). Two were detected on Day 2, a grade 2 and 1, and one was detected on Day 3, a grade 1. Only one positive sample collected on Day 2 post challenge was detected with the ID Screen Influenza A Antigen Capture ELISA. This positive was a grade 3 positive by Directigen Flu A and a grade 2 by Espline Influenza A&B-N and had a titre of $10^{3.25}$ $EID_{50}$. Thus, the only positive sample detected with the ELISA was from a pony shedding a high concentration of virus (Table 4.3).

### 4.1.3 Analytical Sensitivity

Testing of dilutions of allantoic fluid containing cultured virus demonstrated that the most sensitive commercial kit was Directigen Flu A (Table 4.4). This kit detected 0.55 HA units of virus. The colour intensity of Directigen Flu A results decreased with the dilutions of virus from grade 3 (55 HA units) to grade 2 (5.5 HA units) and to grade 1 (0.55 HA units). The limit of detection for Espline Influenza A&B-N and the ID Screen Influenza A Antigen Capture ELISA was 5.5 HA units of virus.

**Table 4. 4 Sensitivity of the antigen detection tests in the detection of cultured EI virus (A/eq/Kildare/89)**

| Dilution | Neat | $10^{-1}$ | $10^{-2}$ | $10^{-3}$ | $10^{-4}$ |
|---|---|---|---|---|---|
| Haemagglutination Units (HA) | 550 | 55 | 5.5 | 0.55 | 0.055 |
| Directigen | Pos 3 | Pos 3 | Pos 2 | Pos 1 | Neg |
| Espline | Pos 3 | Pos 2 | Pos 1 | Neg | Neg |
| Antigen Capture ELISA | Pos | Pos | Pos | Neg | Neg |

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

## 4.1.4 Specificity of Directigen Flu A

It was seen in the earlier tests that although Directigen Flu A was the most sensitive of the antigen detection methods in this study (Figure 4.1, Figure 4.2); it was also the least specific when compared to RT-PCR post challenge (Figure 4.2). Therefore, to further investigate this, a number of different viruses, which had been grown in the laboratory, were tested with Directigen Flu A.

**Table 4. 5 Evaluation of cross-reactivity of Directigen Flu A with Equine Respiratory Viruses**

|  | EHV-1 $TCID_{50} 10^{2..5}$ | EHV-2 $TCID_{50} 10^1$ | EHV-4 $TCID_{50} 10^1$ | Rhino 1 $TCID_{50} 10^7$ | Rhino 2 $TCID_{50} 10^5$ |
|---|---|---|---|---|---|
| **Directigen Flu A** | Neg | Neg | Neg | Neg | Neg |

From the table above (Table 4.5), it can be seen that Directigen Flu A did not cross-react with these other equine respiratory viruses.

#### 4.1.5 Statistical Analysis of Diagnostic Tests Post Natural Infection

Statistical analyses using Cohen's kappa scores (Cohen, 1960) illustrate the agreement between RAD kits, the ID Screen Influenza A Antigen Capture ELISA, VI and RT-PCR.

**Table 4. 6 Cohen's kappa scores for diagnostic methods post natural infection**

| Test 1 | Test 2 | Kappa Scores | SE of Kappa | 95% Confidence Interval | Strength of Agreement |
|--------|--------|--------------|-------------|-------------------------|-----------------------|
| Directigen | Espline | 0.516 | 0.107 | 0.307 to 0.725 | Moderate |
| Directigen | Antigen Capture ELISA | 0.602 | 0.102 | 0.403 to 0.801 | Substantial |
| Directigen | VI | 0.089 | 0.08 | -0.068 to 0.247 | Slight |
| Directigen | RT-PCR | 0.696 | 0.081 | 0.538 to 0.854 | Substantial |
| Espline | Antigen Capture ELISA | 0.681 | 0.121 | 0.443 to 0.918 | Substantial |
| Espline | VI | 0.098 | 0.135 | -0.166 to 0.363 | Slight |
| Espline | RT-PCR | 0.313 | 0.084 | 0.148 to 0.477 | Fair |
| Antigen Capture ELISA | VI | 0.074 | 0.117 | -0.155 to 0.303 | Slight |
| Antigen Capture ELISA | RT-PCR | 0.374 | 0.086 | 0.202 to 0.545 | Fair |
| VI | RT-PCR | 0.064 | 0.044 | 0 to 0.150 | Slight |

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

It can be seen in Table 4.6 that although there was 'substantial' agreement between Directigen Flu A and RT-PCR, and also between Directigen Flu A and the ID Screen Influenza A Antigen Capture ELISA, there was only 'fair' agreement between RT-PCR and the ID Screen Influenza A Antigen Capture ELISA. There was also 'substantial' agreement between Espline Influenza A&B-N and ID Screen Influenza A Antigen Capture ELISA. These two tests had similar sensitivity, less than RT-PCR and Directigen Flu A but more than VI. 'Slight' agreement between VI and the other test methodologies occurred

because of the lack of sensitivity observed with this method when used with samples post natural infection.

**Table 4. 7 Cohen's kappa scores for diagnostic methods post challenge**

| Test 1 | Test 2 | Kappa Scores | SE of Kappa | 95% Confidence Interval | Strength of Agreement |
|---|---|---|---|---|---|
| Directigen | Espline | 0.264 | 0.124 | 0.021 to 0.507 | Fair |
| Directigen | Antigen Capture ELISA | 0.095 | 0.088 | -.079 to .268 | Slight |
| Directigen | VI | 0.332 | 0.091 | 0.153 to 0.511 | Fair |
| Directigen | RT-PCR | 0.221 | 0.072 | 0.079 to 0.362 | Fair |
| Espline | Antigen Capture ELISA | 0.493 | 0.306 | -0.107 to 1.093 | Moderate |
| Espline | VI | 0.102 | 0.056 | -.007 to .211 | Slight |
| Espline | RT-PCR | 0.06 | 0.034 | -.007 to .126 | Slight |
| Antigen Capture ELISA | VI | 0.035 | 0.034 | -.032 to .101 | Slight |
| Antigen Capture ELISA | RT-PCR | 0.02 | 0.02 | -.019 to .059 | Slight |
| VI | RT-PCR | 0.613 | 0.075 | 0.467 to 0.760 | Substantial |

**Directigen= Directigen Flu A, Espline= Espline Influenza A&B-N, Antigen Capture ELISA= ID Screen Influenza A Antigen Capture ELISA, VI=Virus Isolation, RT-PCR=Reverse-Transcription Polymerase Chain Reaction**

In Table 4.7 it is evident that those methods which proved most sensitive (Figure 4.2) in detecting EI virus in challenged animals, have the best agreement. The most sensitive method, RT-PCR only had 'substantial' agreement with VI, but has 'moderate' to 'slight' agreement with all other methods as determined by the kappa score. The least sensitive methods, ID Screen Influenza A Antigen Capture ELISA and Espline Influenza A&B-N had 'moderate' agreement with each other due to the fact that these assays both performed badly post experimental challenge.

## 4.2 Detection of antibodies to Equine Influenza Virus

Introduction

Samples from horses that were initially seronegative by SRH were tested for the detection of seroconversions post natural infection, post challenge and post vaccination.

### 4.2.1 Clinical Samples

Paired samples collected from 203 horses on 14 premises affected by EI were tested by SRH, ID Screen Influenza A Antibody Competition ELISA and HI. Seroconversions are defined as an increase of 50% or $25mm^2$ or greater by SRH, a decrease of 50% or greater competition percentage by ID Screen Influenza A Antibody Competition ELISA and four-fold or greater increase in titre by the HI test. The results are summarised in Figure 4.4 and Table 4.8. Overall the ID Screen Influenza A Antibody Competition ELISA detected fewer seroconversions than the SRH or HI.
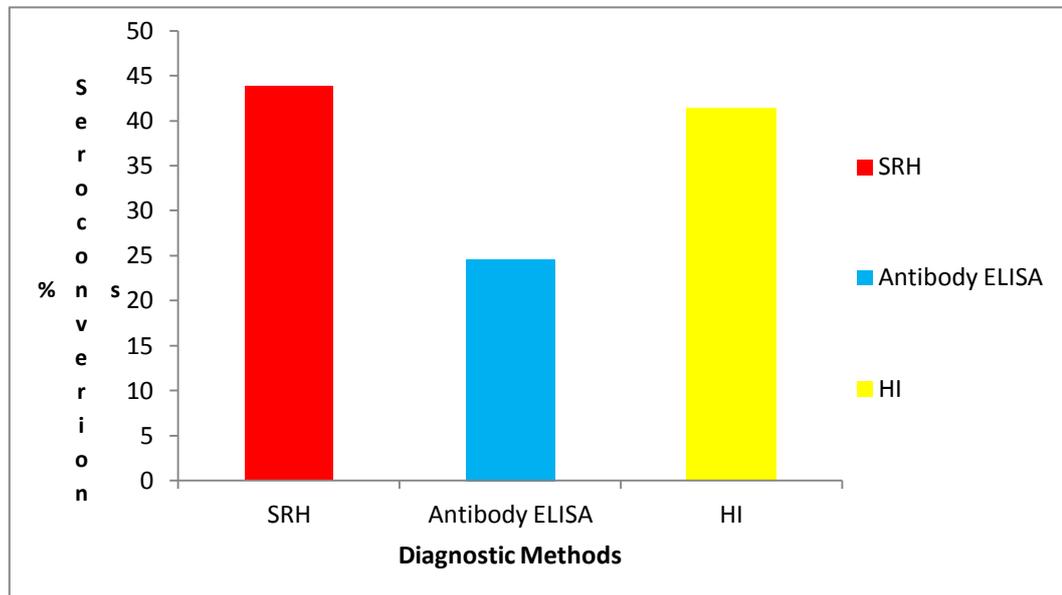


**Figure 4. 4 Percentage of seroconversions detected by SRH, Antibody Competition ELISA or HI in horses (n=203) during outbreaks of EI**

SRH= Single Radial Haemolysis, Antibody ELISA=ID Screen Influenza A Antibody Competition ELISA, HI= Haemagglutination Inhibition

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**Table 4. 8 Number of seroconversions as detected by SRH, Antibody Competition ELISA or HI in seronegative and seropositive horses post natural infection**

| Paired Samples (n=203) | Number SRH Seroconversions | Number Antibody ELISA Seroconversions | Number HI Seroconversions |
|---|---|---|---|
| Total Population | 89/203 | 50/203 | 84/203 |
| Acute SRH Seronegative | 22/28 | 21/28 | 25/28 |
| Acute SRH Seropositive | 67/175 | 29/175 | 59/175 |

**SRH= Single Radial Haemolysis, Antibody ELISA=ID Screen Influenza A Antibody Competition ELISA, HI= Haemagglutination Inhibition**

Seronegative horses that seroconverted by SRH but failed to seroconvert by ID Screen Influenza A Antibody Competition ELISA were initially seropositive by ELISA (n=2; data not shown). Twelve horses that were seropositive by SRH but seronegative by ELISA all seroconverted by ELISA and 11 seroconverted by SRH. These results suggest that the ability of a particular test to detect a seroconversion is related to the initial serological status as determined by that specific test.

**4.2.1.1 Statistical Analysis of Diagnostic Tests Post Natural Infection**

**Table 4. 9 Cohen's kappa scores for diagnostic methods post natural infection**

| Test 1 | Test 2 | Kappa Scores | SE of Kappa | 95% Confidence Interval | Strength of Agreement |
|---|---|---|---|---|---|
| SRH | Antibody ELISA | 0.550 | 0.071 | 0.417 to 0.695 | Moderate |
| SRH | HI | 0.775 | 0.058 | 0.662 to 0.889 | Substantial |
| Antibody ELISA | HI | 0.642 | 0.066 | 0.512 to 0.771 | Substantial |

**SRH= Single Radial Haemolysis, Antibody ELISA=ID Screen Influenza A Antibody Competition ELISA, HI= Haemagglutination Inhibition**

The SRH test, the test which detected the greatest number of seroconversions and the HI test, the test with the second greatest number of seroconversions had 'substantial' agreement in detecting seroconversions in post natural infection samples. Both of these tests had 'substantial' agreement with acute samples and convalescent samples as indicated by Table 4.9. However, only 'moderate' agreement was detected between the ID Screen Influenza A Antibody Competition ELISA and the SRH test. This was due mainly to the fact that in seronegative samples, the ID Screen Influenza detected more acute positives than the SRH, and in the seropositive samples, the ID Screen Influenza A Antibody Competition ELISA detected more acute seronegatives than the SRH test. The HI was in agreement with the ID Screen Influenza A Antibody Competition ELISA in some cases and both tests differed from the SRH test. Therefore a 'substantial' agreement was also seen between HI and ELISA tests.

## 4.2.2 Detection of Equine Influenza Antibodies in Samples Post Challenge

Fourteen seronegative foals were experimentally challenged with EI virus and serologically monitored as described previously. The percentage of seroconversions detected by SRH and ID Screen Influenza A Antibody Competition ELISA on each day of sampling is summarised in Figure 4.5.
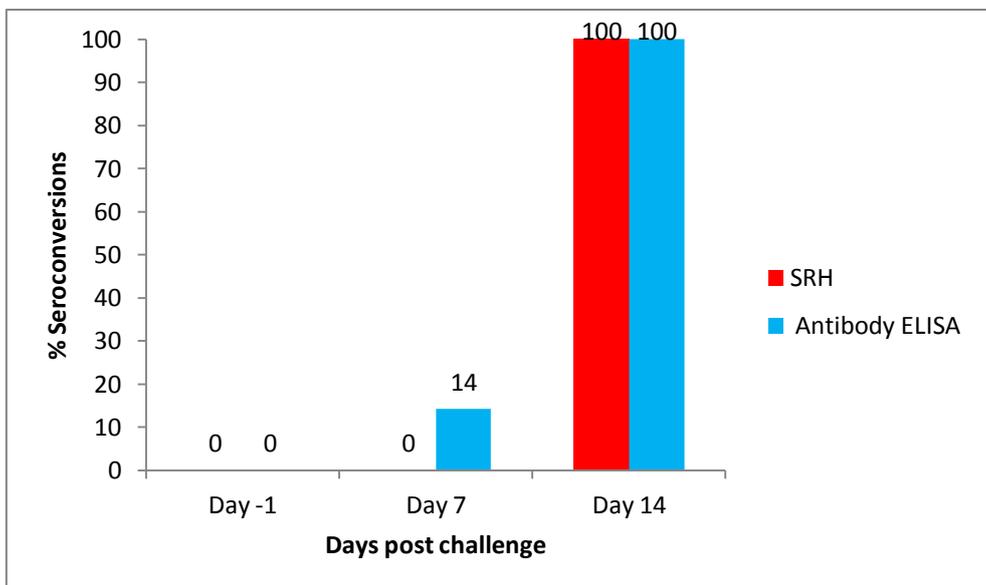


**Figure 4. 5 Percentage seroconversions detected by SRH and Antibody Competition ELISA post challenge (n=14)**

**SRH= Single Radial Haemolysis, Antibody ELISA=ID Screen Influenza A Antibody Competition ELISA**
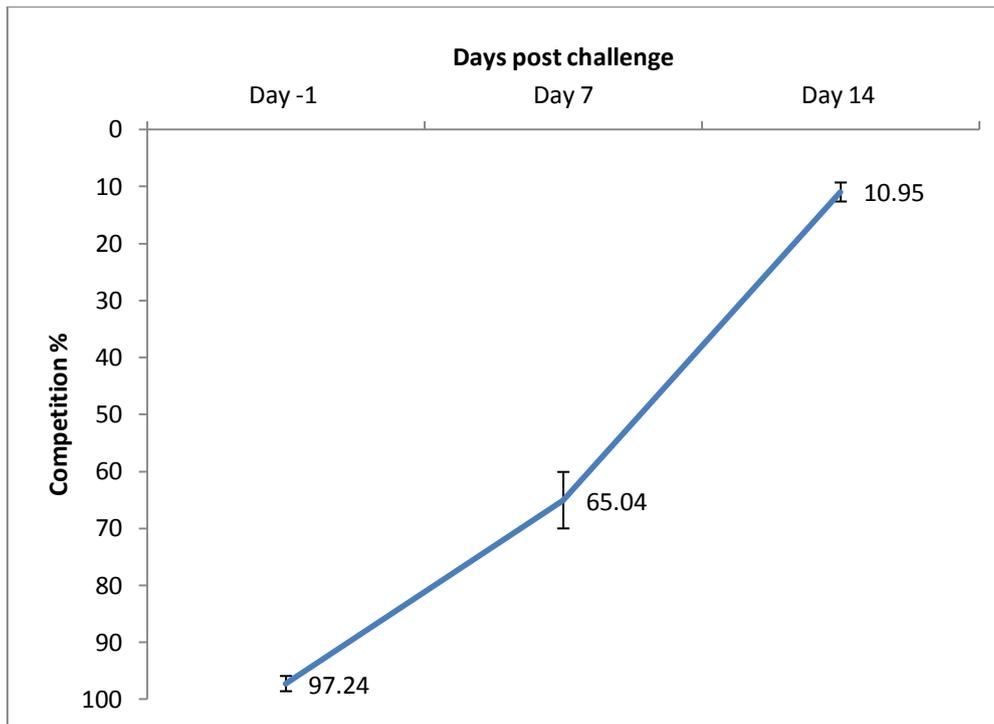
**Figure 4. 6 Decrease in competition percentage in samples collected post challenge as measured by the Antibody Competition ELISA (n=14)**

**The y axis values for Figure 4.6 are reversed.**

All the foals were seronegative on Day -1 i.e. prior to challenge. All foals had seroconverted by SRH by Day 14 post challenge, but none had seroconverted by Day 7 post challenge. The ID Screen Influenza A Antibody Competition ELISA also detected that all horses had seroconverted by 14 days post challenge and that two of these had seroconverted by 7 days post challenge (Figure 4.5). Ten other horses exhibited antibody responses detectable by ID Screen Influenza A Antibody Competition ELISA by 7 days post challenge although these responses were not of sufficient magnitude to be designated seroconversions (Figure 4.6). This was in contrast to the SRH which did not detect an antibody response in any horse by day 7 (Figure 4.5).

## 4.2.2.1 Statistical Analysis of Diagnostic Tests Post Challenge

A comparison was made between the levels of agreement of the SRH assay and the ID Screen Influenza A Antibody Competition ELISA. The kappa score was 0.897 (CI 0.757 to 1.036), (SE $\pm$ 0.071). This strength of agreement was considered 'almost perfect'. Both SRH and ID Screen Influenza A Antibody

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Competition ELISA were in agreement at Day -1 and Day 14 when 100% tested negative and positive respectively by both tests.

**4.2.3 Serological response of Seronegative Weanlings Post Vaccination as detected by SRH and ID Screen Influenza A Antibody Competition ELISA**

Samples from 60 weanlings that had participated in a comparative vaccine study were tested by ID Screen Influenza A Antibody Competition ELISA and the results compared to those previously obtained by SRH (Gildea et al., 2011a). Weanlings were randomly allocated to receive one of the five vaccines which were administered on 3 occasions, five weeks apart followed by a third dose six months later. All weanlings were seronegative by SRH at the time of first vaccination. Their antibody responses as measured by SRH and ID Screen Influenza A Antibody Competition ELISA are illustrated in Figure 4.7. The SRH results have been published previously (Gildea et al., 2011a) and are represented here for comparative purposes.

*
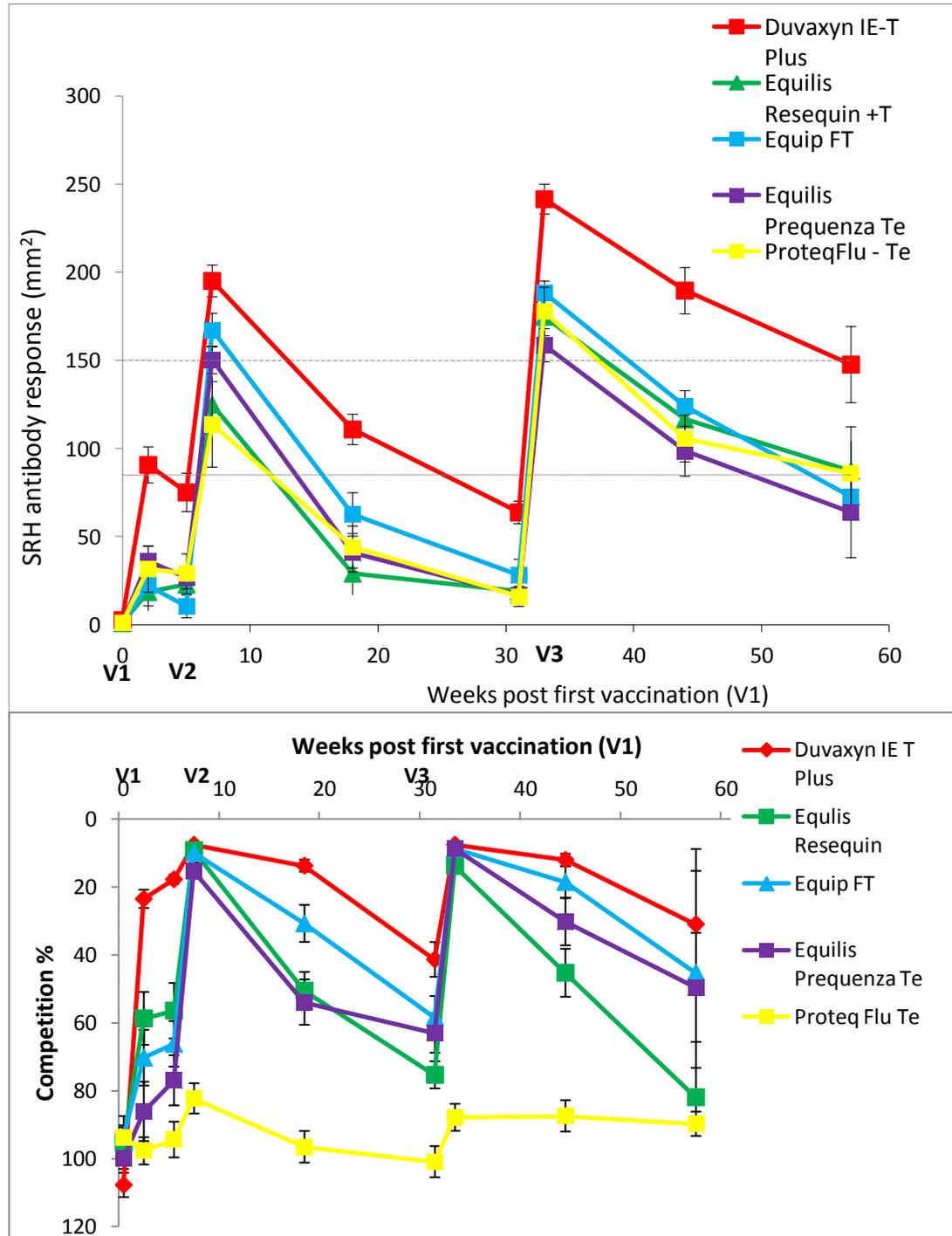


**Figure 4. 7  Mean anti-HA and anti-NP antibody levels post EI vaccination as measured by SRH (upper panel) and Antibody Competition ELISA (lower panel) (n=60)**

*SRH graph has been published (Gildea et al., 2011c) and is shown here for comparative purposes

In Figure 4.7 it can be seen that the pattern of antibody response post vaccination detected by ID Screen Influenza A Antibody Competition ELISA was similar to that detected by the SRH test for 4 of the vaccines. The ID Screen Influenza A Antibody Competition ELISA did not detect an antibody response in weanlings vaccinated with Proteq Flu Te indicating potential usefulness of the combination of this kit and vaccine in a "DIVA" control strategy. The antibody response to the other 2 subunit vaccines Equip FT and Equilis Prequenza Te was detected by ID Screen Influenza A Antibody Competition ELISA, i.e. no DIVA capacity was evident. The ID Screen Influenza A Antibody Competition ELISA demonstrated similar sensitivity to the SRH in detecting a higher and more persistent antibody response in horses vaccinated with Duvaxyn IE T Plus than other vaccines at V+2 weeks, V2, V2+13 weeks, V3 and V3+13 weeks ($p < 0.005$). Also, the ID Screen Influenza A Antibody Competition ELISA was consistent with the SRH in detecting a similar response to Equilis Resequin, Equip FT and Equilis Prequenza Te with only minor differences in the magnitude of responses to these vaccines ($p > 0.05$).
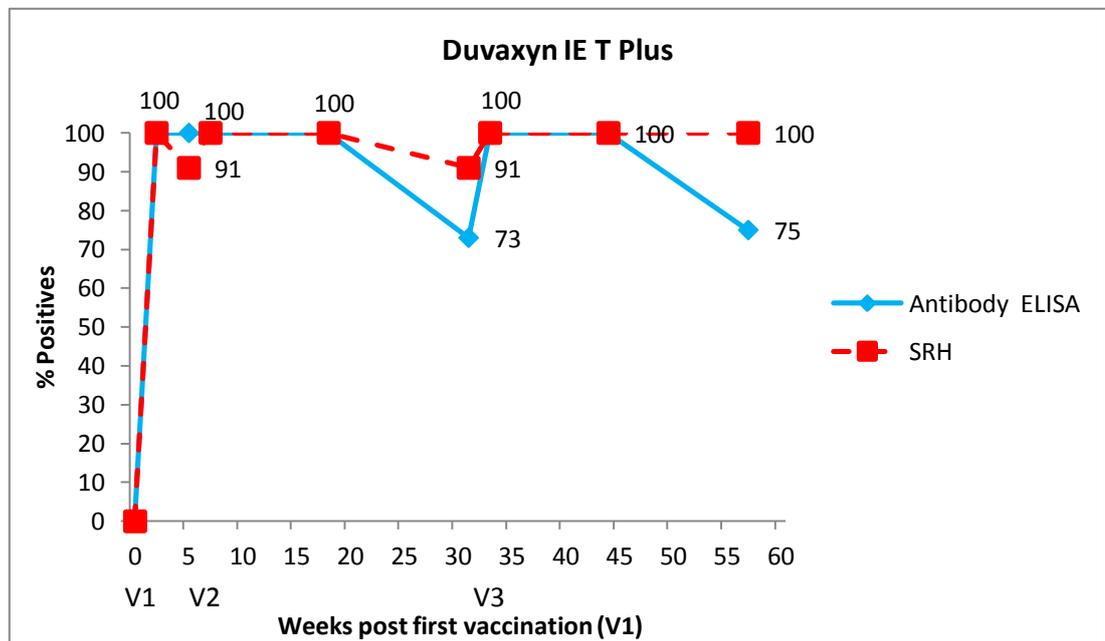


**Figure 4. 8 Percentage of EI positive horses detected by Antibody Competition ELISA or SRH following vaccination with Duvaxyn IE T Plus**

Antibody ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH=Single Radial Haemolysis
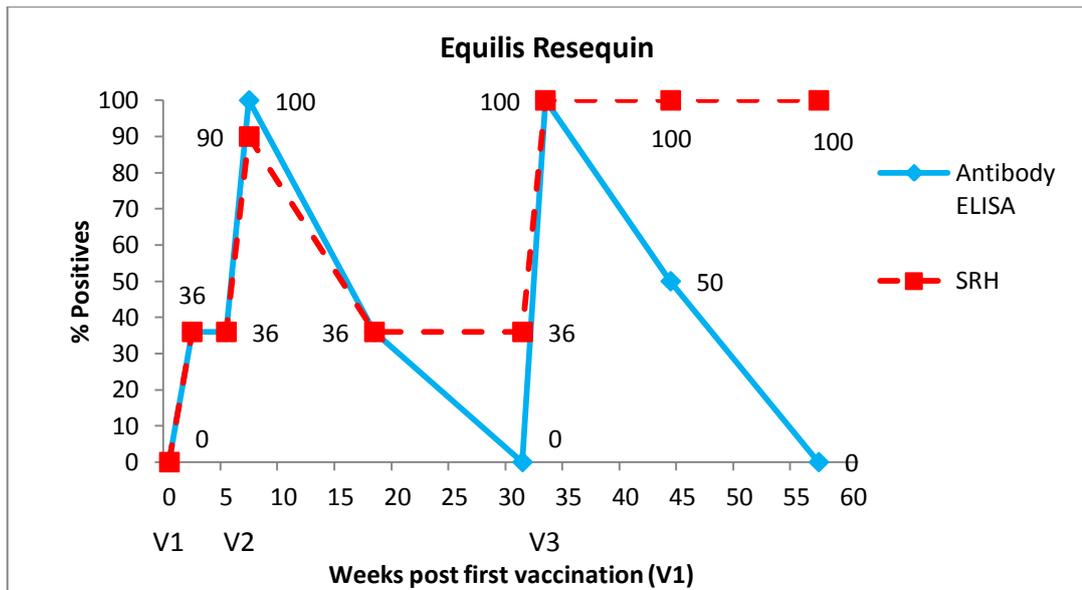
**Figure 4. 9 Percentage of EI positive horses detected by Antibody Competition ELISA or SRH following vaccination with Equilis Resequin**

**Antibody ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH=Single Radial Haemolysis**
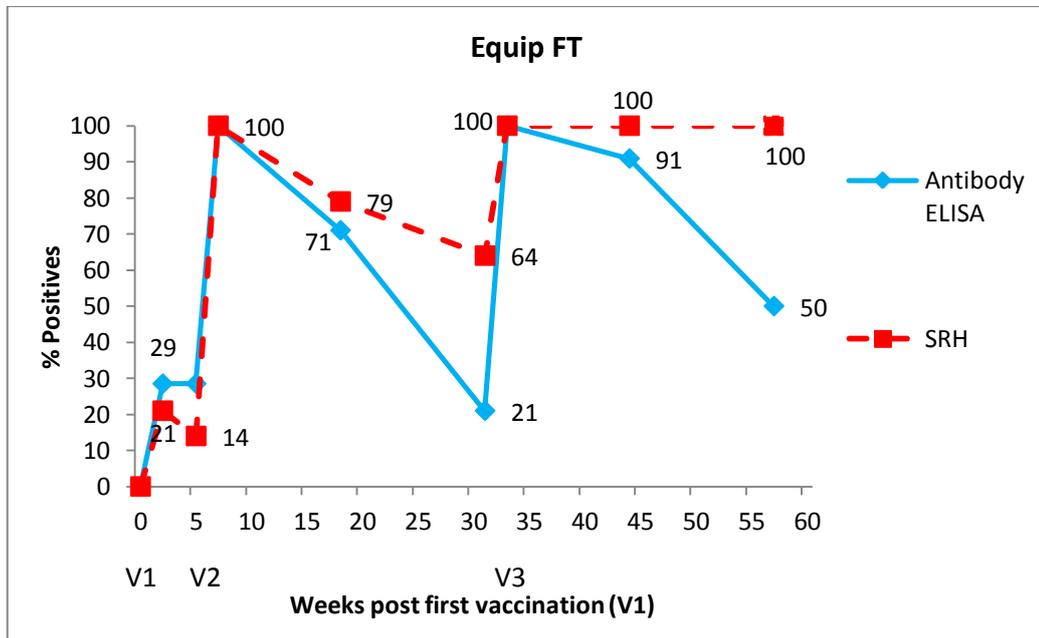


**Figure 4. 10 Percentage of EI positive horses detected by Antibody Competition ELISA or SRH following vaccination with Equip FT**

**Antibody ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH=Single Radial Haemolysis**
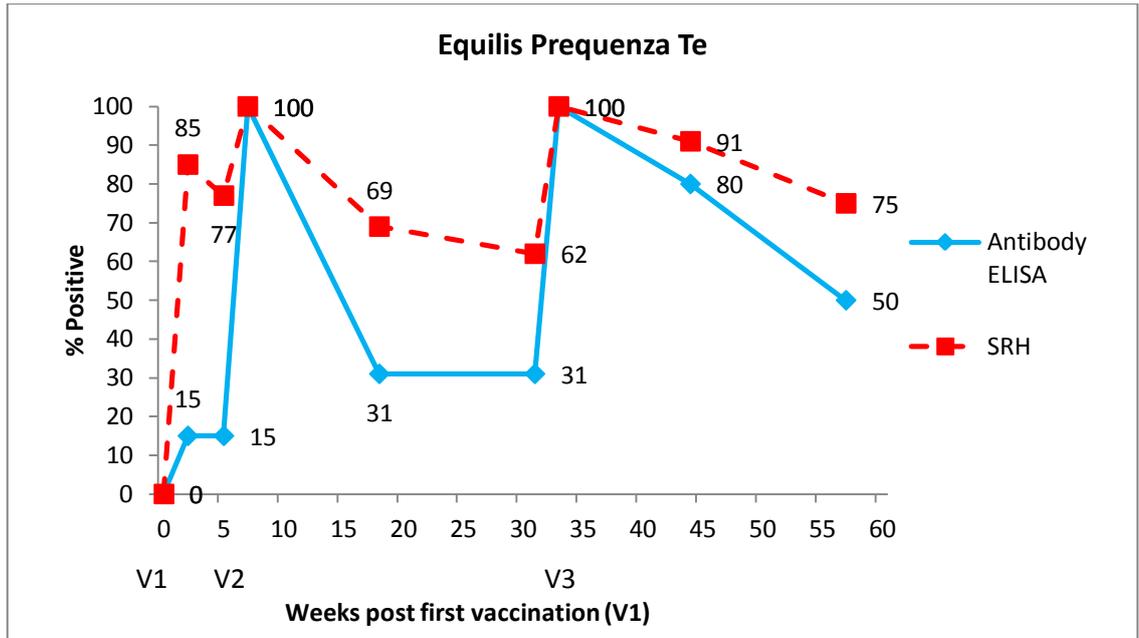
**Figure 4. 11 Percentage of EI positive horses detected by Antibody Competition ELISA or SRH following vaccination with Equilis Prequenza Te**

**Antibody ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH=Single Radial Haemolysis**
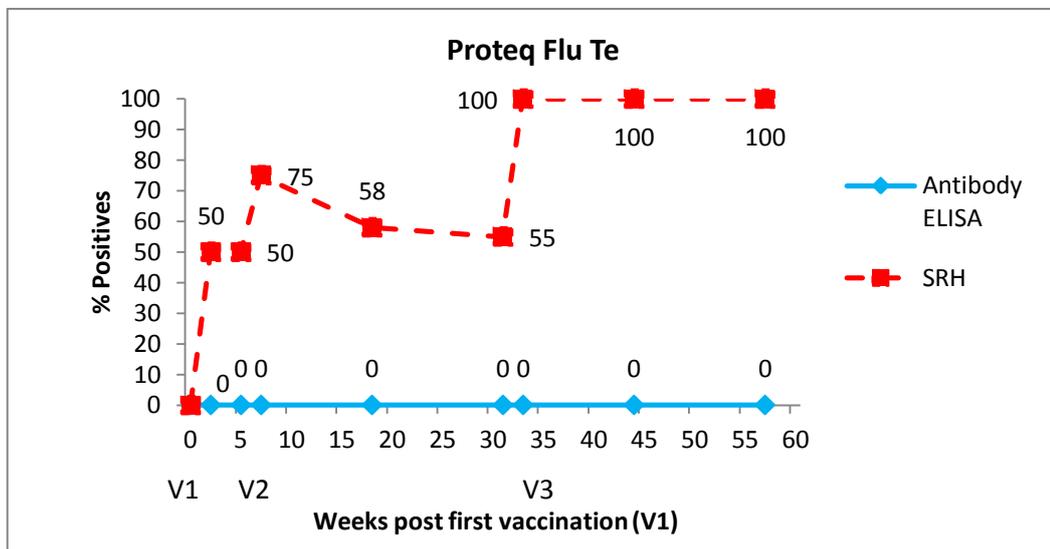


**Figure 4. 12 Percentage of EI positive horses detected by Antibody Competition ELISA or SRH following vaccination with Proteq Flu Te**

**Antibody ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH=Single Radial Haemolysis**

**Antibody Response to Duvaxyn IE T Plus**

At the time of V1, all of the eleven weanlings vaccinated with the whole inactivated vaccine Duvaxyn IE T Plus were seronegative by both SRH and ID Screen Influenza A Antibody Competition ELISA (Figure 4.8). From two weeks post V1 until the end of the study 91–100% of the horses were seropositive by SRH and 73–100% were positive by ID Screen Influenza A Antibody Competition ELISA. The decreases in the number of seropositives were observed for both assays at the time of V2 and V3 and for the ID Screen Influenza A Antibody Competition ELISA; at the last sampling time point when only 4 horses were available for sampling. The antibodies detected by ID Screen Influenza A Antibody Competition ELISA were generally more persistent in this group than in any of the other vaccine groups ($p<0.005$ at V+2weeks, V2, V+13 weeks, V3 and V3+13 weeks).

**Antibody Response to Equilis Resequin**

It can be seen from Figure 4.9 that all eleven weanlings vaccinated with the whole inactivated vaccine Equilis Resequin were seronegative by the ID Screen Influenza A Antibody Competition ELISA and SRH at V1. There was good agreement between the sensitivity of the two tests after the first two doses of vaccine. At 2 weeks and 5 weeks post V1 (time of V2) 36% of the horses were positive by both ID Screen Influenza A Antibody Competition ELISA and SRH. The SRH and the ID Screen Influenza A Antibody Competition ELISA detected 90% and 100% of the horses positive at V2+2 weeks respectively and both detected 36% of the horses as positive at 13 weeks post V2. At the time of V3 i.e. 26 weeks post V2, 36% were positive by SRH but none of the horses were positive by ELISA. Two weeks post V3, 100% were positive by SRH and remained so up to the completion of the study at 26 weeks post V3. However, although 100% of the horses were positive by ID Screen Influenza A Antibody Competition ELISA 2 weeks post V3, this had declined to 50% by 13 weeks post V3 and to 0% by 26 weeks post V3. However, at V3 +13 weeks, only ten weanlings were available for sampling and at V3 + 26 weeks, four weanlings were available.

**Antibody Response to Equip FT**

All of the 14 weanlings vaccinated with the subunit vaccine Equip FT were seronegative at V1 as determined by both ID Screen Influenza A Antibody Competition ELISA and SRH (Figure 4.10). The agreement between the two tests was consistent with that observed in the group vaccinated with Equilis Resequin i.e. the sensitivity was very similar up to 13 weeks post V2 but by V3, 64% of the horses were positive by SRH as compared to 21% by ID Screen Influenza A Antibody Competition ELISA. Two weeks post V3, 100% of the weanlings were positive by both assays and remained so by SRH up to the end of the study at 26 weeks post V3. At 13 weeks post V3, 91% were positive by ID Screen Influenza A Antibody Competition ELISA. Only two weanlings were available for sampling at the end of the study i.e. 26 weeks post V3.

**Antibody Response to Equilis Prequenza Te**

The 13 weanlings vaccinated with the subunit vaccine Equilis Prequenza Te were seronegative by both assays at the time of V1 (Figure 4.11). The ID Screen Influenza A Antibody Competition ELISA response to V1 resembled that of the group vaccinated with the other subunit vaccine Equip FT ($p>0.05$). Only 15% of the horses were seropositive by ID Screen Influenza A Antibody Competition ELISA at V1+2 weeks and V1+5 weeks. As with all the vaccines except Proteq Flu Te, all of the horses seroconverted as determined by ID Screen Influenza A Antibody Competition ELISA by two weeks post V2 and two weeks post V3. The decline in antibodies detected by the ID Screen Influenza A Antibody Competition ELISA after V2 was similar to that observed in the group vaccinated with Equilis Resequin. At 13 weeks post V2 and at the time of V3 only 31% of the horses were seropositive by ID Screen Influenza A Antibody Competition ELISA ($p>0.05$). At 13 weeks and 26 weeks post V3, 80% and 50% respectively were positive by ID Screen Influenza A Antibody Competition ELISA.

**Antibody Response to Proteq Flu Te**

At the time of V1, all 11 weanlings vaccinated with the canary pox recombinant vaccine Proteq Flu Te were seronegative by both SRH and ID Screen Influenza A Antibody Competition ELISA (Figure 4.12). The pattern of the SRH response to vaccination was comparable to that of the groups vaccinated with the other vaccines but the ELISA did not detect antibodies against influenza at any stage of sampling.

**Failure or delay in H3N8 response to vaccination**

Some horses did not respond initially to vaccination as detected by SRH (Gildea et al., 2011c) and/or ID Screen Influenza A Antibody Competition ELISA. These data are summarised in Table 4.10. The weanlings with maternally derived antibodies (MDAs) were excluded from this analysis as were the weanlings vaccinated with Proteq Flu Te.

**Table 4. 10 Number of weanlings which failed to seroconvert by SRH or Antibody Competition ELISA**

| Vaccine | (S2) | | (S3) | | (S4) | | (S7) | |
|---|---|---|---|---|---|---|---|---|
| | SRH | E* | SRH | E | SRH | E | SRH | E |
| Duvaxyn IE T Plus | 0/12 (0%) | 0/11 (0%) | 0/12 (0%) | 0/11 (0%) | 0/12 (0%) | 4/11 (36%) | 0/12 (0%) | 0/11 (0%) |
| Equilis Resequin | 7/11 (64%) | 7/11 (64%) | 6/11 (55%) | 6/11 (55%) | 1/11 (9%) | 0/11 (0%) | 0/11 (0%) | 0/11 (0%) |
| Equip FT | 11/14 (79%) | 10/14 (71%) | 11/14 (79%) | 9/14 (64%) | 0/14 (0%) | 0/14 (0%) | 0/14 (0%) | 1/14 (7%) |
| Prequenza Te | 1/13 (8%) | 11/13 (85%) | 1/13 (8%) | 10/13 (77%) | 0/13 (0%) | 0/13 (0%) | 0/13 (0%) | 0/13 (0%) |

S2 = two weeks post V1; S3 = at the time of V2; S4 = two weeks post V2; S7 = two weeks post V3; SRH=Single Radial Haemolysis, E = ID Screen Influenza A Antibody Competition ELISA ; *N=11, two weanlings were identified as having MDA by ELISA but only one was previously identified as having MDA by SRH (Gildea et al., 2011c).

The SRH and the ID Screen Influenza A Antibody Competition ELISA failed to detect seroconversions in 19 and 32 cases post vaccination respectively (Table 4.10). The number of weanlings vaccinated with Equilis Resequin and Equip FT that failed to seroconvert post V1 was similar with both the SRH and ID Screen Influenza A Antibody Competition ELISA assay. However, 85% of the

weanlings vaccinated with Equilis Prequenza Te failed to seroconvert by ID Screen Influenza A Antibody Competition ELISA but only eight per cent failed to seroconvert by SRH. At S4, 36% of horses vaccinated with Duvaxyn IE T Plus failed to seroconvert by ID Screen Influenza A Antibody Competition ELISA but these horses were strongly seropositive by ID Screen Influenza A Antibody Competition ELISA at the time of S3 (9.38% to 16.55%, data not shown). In contrast, one seronegative (competition percentage-53.00%) horse vaccinated with Equilis Resequin failed to seroconvert by SRH but seroconverted by ID Screen Influenza A Antibody Competition ELISA. One horse failed to seroconvert to V3 by ID Screen Influenza A Antibody Competition ELISA but was strongly positive at the time of vaccination (competition percentage-16.42%).

Table 4.11 illustrates the ID Screen Influenza A Antibody Competition ELISA and SRH results of poor responders to vaccination. Weanlings with MDAs were analysed separately from weanlings that were seronegative at the time of V1. None of the weanlings were seropositive by both assays at the time of V1. Only one weanling was seropositive by ID Screen Influenza A Antibody Competition ELISA. On vaccination with Duvaxyn IE T Plus, no ID Screen Influenza A Antibody Competition ELISA response was detected after V1 in this animal but the weanling seroconverted to V2 and V3. Post V2 and V3, the weanling mounted an ELISA response similar to that of the other weanlings that were seronegative at the time of V1.The weanling seroconverted by SRH after each vaccination. Poor responders by ID Screen Influenza A Antibody Competition ELISA to V1 were detected in all vaccine groups but irrespective of the vaccine used, the mean ELISA response to V2 and V3 was similar to that of the weanlings that seroconverted to V1.

**Table 4. 11 Mean H3N8 antibody levels (SRH=mm$^2$) and mean competition percentage (Antibody Competition ELISA) for weanlings with MDA, poor responders or normal weanlings**

| Vaccine | V1 | S2 | V2 | S4 | S5 | V3 | S7 | S8 | S9* |
|---|---|---|---|---|---|---|---|---|---|
| **Duvaxyn IET Plus** | | | | | | | | | |
| MDA SRH (1) | 39.82 | 36.83 | 28.23 | 142.22 | 59.24 | 14.14 | 240.29 | 187.87 | N/A |
| MDA ELISA (1) | 29.81 | 33.98 | 42.78 | 4.34 | 12.28 | 50.54 | 4.34 | 9.87 | 13.58 |
| Poor responders SRH (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders ELISA (1) | 88.62 | 46.28 | 60.43 | 9.33 | 19.78 | 55.27 | 8.83 | 14.50 | N/A |
| Normal SRH (12) | 1.17 | 94.67 | 81.51 | 206.36 | 111.85 | 63.26 | 247.07 | 192.8 | 150.92 |
| Normal ELISA (11) | 107.81 | 21.48 | 17.82 | 7.62 | 13.80 | 41.40 | 7.60 | 12.08 | 31.81 |
| | | | | | | | | | |
| **Equilis Resequin** | | | | | | | | | |
| MDA SRH (2) | 53.94 | 37.26 | 32.84 | 32.83 | 0.00 | 0.00 | 128.35 | 67.05 | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (7) | 1.02 | 10.62 | 22.01 | 108.50 | 14.68 | 5.45 | 171.53 | 111.33 | 98.52 |
| Poor responders ELISA (9) | 91.21 | 77.80 | 67.50 | 9.79 | 54.41 | 79.10 | 13.26 | 43.63 | 81.96 |
| Normal SRH (4) | 0.00 | 59.81 | 45.57 | 165.61 | 69.06 | 44.37 | 185.93 | 125.75 | 74.07 |
| Normal ELISA (4) | 93.76 | 30.61 | 32.00 | 9.72 | 46.95 | 70.71 | 13.82 | 44.52 | N/A |
| | | | | | | | | | |
| **Equip FT** | | | | | | | | | |
| MDA SRH (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (11) | 0.52 | 6.15 | 5.03 | 167.38 | 54.74 | 28.04 | 197.97 | 129.55 | 76.82 |
| Poor responders ELISA (10) | 92.08 | 83.87 | 76.70 | 11.85 | 39.45 | 65.42 | 9.07 | 23.96 | 45.29 |
| Normal SRH (3) | 0 | 119.07 | 67.48 | 215.23 | 103.14 | 43.44 | 193.14 | 123.23 | N/A |
| Normal ELISA (4) | 98.11 | 36.47 | 40.13 | 8.45 | 18.13 | 41.00 | 8.06 | 9.24 | N/A |
| | | | | | | | | | |
| **Equilis Prequenza Te** | | | | | | | | | |
| MDA SRH (1) | 53.53 | 55.77 | 43.29 | 128.53 | 41.11 | 20 | 165.96 | 116.15 | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (1) | 0 | 6.07 | 5.44 | 123.8 | 6.09 | 5.67 | 89.45 | 16.64 | 6.52 |
| Poor responders ELISA (12) | 100.17 | 94.61 | 81.39 | 16.47 | 57.68 | 66.41 | 9.23 | 33.94 | 53.25 |
| Normal SRH (12) | 0 | 44.6 | 30.17 | 161.32 | 51.92 | 19.59 | 169.43 | 109.43 | 84.94 |
| Normal ELISA (2) | 88.80 | 37.08 | 45.60 | 13.08 | 41.69 | 53.69 | 5.38 | 9.22 | 38.79 |

**Poor responders in Table 4.11 included weanlings that failed to seroconvert to V1 only as detected by ELISA.  S2 = 2 weeks post V1; S4 = 2 weeks post V2; S5 = 3 months post V2; S7 = 2 weeks post V3; S8 = 3 months post V3; S9 = 6 months post V3. SRH data taken from Gildea et al., 2011c MDA= Maternally Derived Antibodies, ELISA= ID Screen Influenza A Antibody Competition ELISA, SRH= Single Radial Haemolysis, SE added in Appendix**

#### 4.2.3.1 Statistical Analysis of Diagnostic Tests for Seronegative Samples Post Vaccination

The comparison between the SRH assay and the ID Screen Influenza A Antibody Competition ELISA gave a kappa score value of 0.535 (CI 0.453 to 0.617, SE $\pm$ 0.042). These results were calculated without including results from Proteq Flu Te. The strength of agreement between the tests for the other four vaccines was considered to be 'moderate'. Proteq Flu Te when used with the ID Screen Influenza A Antibody Competition ELISA is a good vaccine for DIVA. The SRH however can detect antibodies to this vaccine, and therefore, the assays do not give similar results in this case

#### 4.2.4 Seropositive Samples Post Vaccination as determined by SRH and ID Screen Influenza A Antibody Competition ELISA

Samples from 44 horses that had participated in a comparative vaccine study following annual booster vaccination were tested by ID Screen Influenza A Antibody Competition ELISA and the results compared to those obtained by SRH. The results are illustrated in Figure 4.13. The SRH results have been published previously (Gildea et al., 2011b) and are presented here for comparison purposes. No increase in antibodies was detected by ID Screen Influenza A Antibody Competition ELISA in the horses vaccinated with Proteq Flu Te as the ID Screen Influenza A Antibody Competition ELISA detects antibodies against the nucleocapsid, and not the canary pox vector in Proteq Flu Te expressing only the haemagglutinin protein of EI. No significant differences between the antibody responses induced at two weeks following booster vaccination with any of the other five vaccines was detected by ID Screen Influenza A Antibody Competition ELISA ($p > 0.05$). The antibody profiles against the nucleocapsid followed a similar pattern to the antibodies against the haemagglutinin in that they peaked two weeks post vaccination (mean competition percentage- 11.13%, SE$\pm$ 1.588), decreased by three months post vaccination (mean competition percentage-20.47%, SE$\pm$3.041) and then the majority of horses declined to near their original levels (mean competition percentage- 30.38%, SE $\pm$4.972) by six months post vaccination (mean competition percentage-26.23%, SE $\pm$4.34).
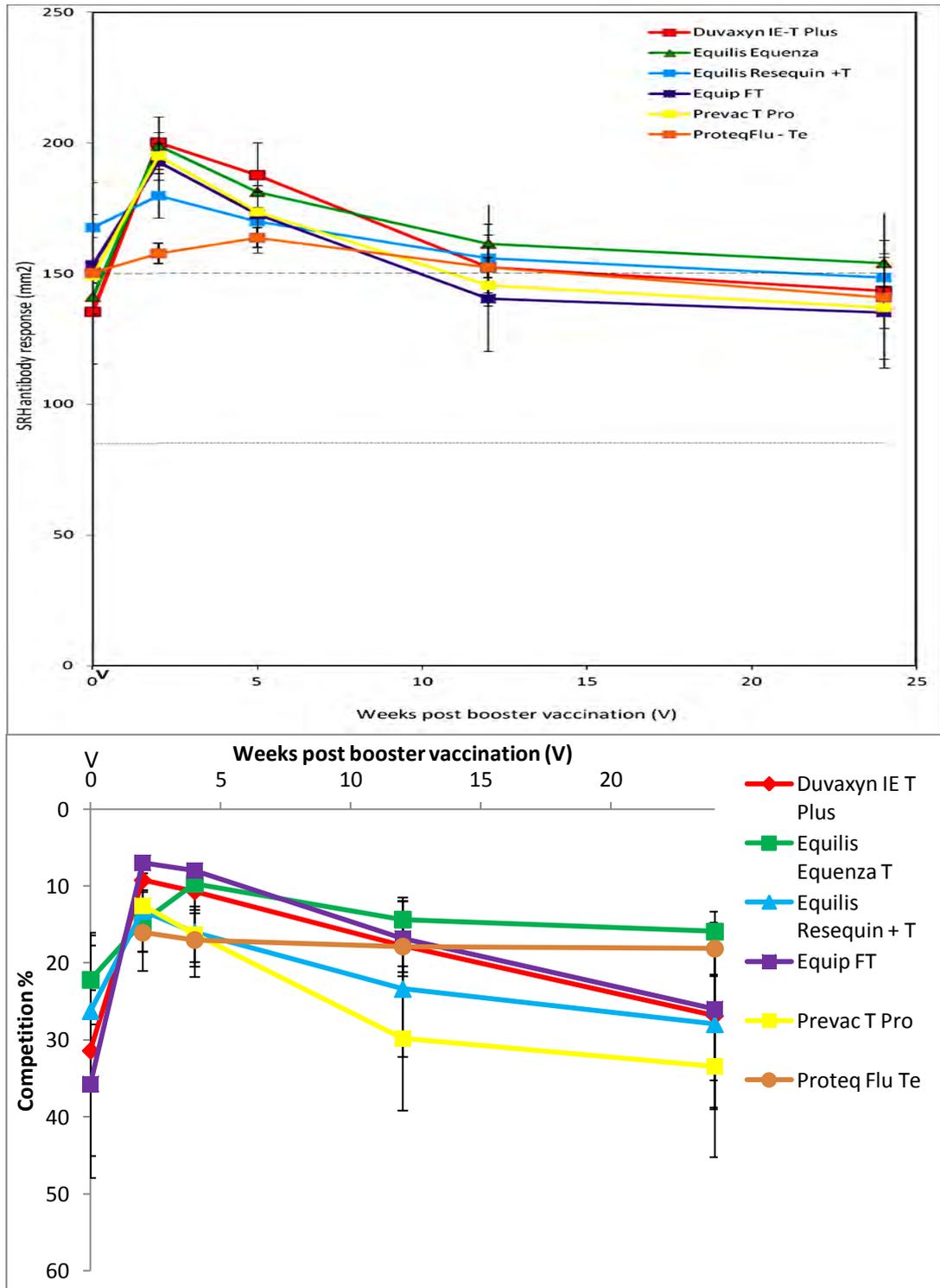
**Figure 4. 13 Mean SRH antibody (upper level) and Antibody Competition ELISA (lower level) results post booster vaccination (n=44)**

*SRH graph has been published (Gildea et al., 2011b) and is shown here for comparative purposes

All of the horses remained positive by SRH for the duration of the study with the exception of one horse that tested negative by three months post vaccination (Gildea et al., 2011b). This horse also tested negative by ID Screen Influenza A Antibody Competition ELISA three months post booster vaccination, as did four other horses. However, the SRH levels for these four horses at that time point varied from 82 mm$^2$–157 mm$^2$. Three additional horses tested negative by ID Screen Influenza A Antibody Competition ELISA six months post booster vaccination when their SRH levels varied from 60 mm$^2$–149 mm$^2$. Six months post vaccination five horses had SRH levels >85 mm$^2$, i.e. below that required for clinical protection and one horse was seronegative (Gildea et al., 2011b). Only one of these horses tested positive by ID Screen Influenza A Antibody Competition ELISA at this time point.

Eighteen of the 44 horses (41%) included in the original study did not demonstrate a significant rise in SRH level to H3N8 (mean SRH levels against A/eq/Newmarket/2/93, A/eq/Kildare/92, A/eq/South Africa/4/03) post booster vaccination (Gildea et al., 2011b). Excluding the horses vaccinated with Proteq Flu Te, 22 of the 38 horses (58%) did not seroconvert by ID Screen Influenza A Antibody Competition ELISA and 13 (34%) did not seroconvert by SRH. All of the horses that did not seroconvert by ID Screen Influenza A Antibody Competition ELISA had a competition percentage of <25% at the time of vaccination. The mean acute ID Screen Influenza A Antibody Competition ELISA competition percentage of the horses which did not seroconvert post booster vaccination was 12.79% (SE±2.456) compared to a mean competition percentage of 54.56% (SE±8.118) for the horses that did seroconvert. Eleven horses that did not seroconvert by ID Screen Influenza A Antibody Competition ELISA seroconverted by SRH. These horses had a mean H3N8 SRH level of 146.79mm$^2$ (SE±7.017) at the time of vaccination. Their mean competition percentage was 9.52% (SE±0.788). Of the 13 horses that did not seroconvert by SRH, three of them seroconverted by ID Screen Influenza A Antibody Competition ELISA. The mean H3N8 SRH antibody level of these three horses was 200.87mm$^2$ (SE±13.252). Their mean competition percentage was 44.64% (SE±24.867). One of these horses tested negative by ID Screen Influenza A Antibody Competition ELISA at the time of vaccination.

**Table 4. 12 Influence of pre-existing H3N8 antibody levels on booster vaccination response**

\*

| Existing SRH H3N8 levels at time of Vaccination | Increase in SRH level of $\geq$ 25 mm$^2$ | Increase in SRH level of < 25 mm$^2$ |
|---|---|---|
| <50 mm$^2$ | 3/3 | 0/3 |
| >50 mm$^2$ < 85 mm$^2$ | 6/6 | 0/6 |
| >85 mm$^2$ < 150 mm$^2$ | 13/15 | 2/15 |
| > 150 mm$^2$ | 3/14 | 11/14 |
| Total | 25/38 | 13/38 |

| Existing ELISA H3N8 levels at time of Vaccination | Decrease of $\geq$50% | Decrease of <50% |
|---|---|---|
| > 85% | 3/3 | 0/3 |
| >50% <85% | 5/6 | 1/6 |
| >15% <50% | 7/9 | 2/9 |
| <15% | 1/20 | 19/20 |
| Total | 16/38 | 22/38 |

**Excluding horses vaccinated with Proteq Flu Te**

**\*SRH Table has been published (Gildea et al., 2011b) and is shown here for comparative purposes**

Table 4.12 summarises the effect of pre-existing antibody levels on the response to booster vaccination. The ID Screen Influenza A Antibody Competition ELISA test was similar to the SRH in that the pre-existing antibody levels correlated significantly with response to vaccination (p<0.05). Horses with low antibody levels, competition percentage >15% responded best to vaccination .i.e. 15 of 18 seroconverted. Only one of 20 horses with a competition percentage <15 seroconverted.

### 4.2.4.1 Statistical Analysis of Diagnostic Tests for Seropositive Samples Post Vaccination

The kappa score calculated was 0.146 (CI -0.035 to 0.326, SE $\pm$ 0.092). This indicated a 'slight' agreement between the SRH and the ID Screen Influenza A Antibody Capture ELISA in post vaccination samples.

## 4.3 Detection of antibodies to Equine Influenza H7N7

### 4.3.1 Seroconversion to H7N7 following exposure to H3N8 virus

Paired serum samples collected from horses that had seroconverted to EI (H3N8), during confirmed outbreaks in 2009 and 2010 were tested by the ID Screen Influenza A H7 Antibody Competition ELISA to determine if cross reactivity to H7N7 and H3N8 is encountered with this assay. The seroconversions to H3N8 were previously detected by both SRH and HI. There was no history of vaccination during the outbreaks.
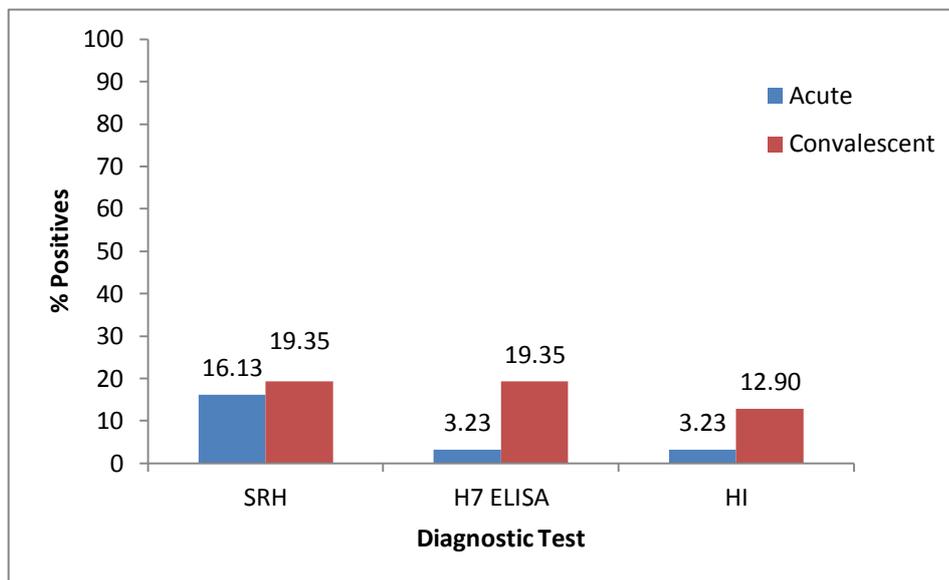


**Figure 4. 14 Percentage of H7N7 EI virus positives pre and post natural infection (n=31)**

**H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis, HI= Haemagglutination Inhibition**

Five of the horses (16.13%) were seropositive for H7N7 by SRH on initial sampling, and 6 (19.35%) were seropositive at the convalescent stage (Figure 4.14). Two horses seroconverted to H7N7 by SRH during the outbreak.

One of the horses (3.23%) was seropositive for H7N7 by HI on initial sampling, and 4 (12.9%) were seropositive at the convalescent stage (Figure 4.14). Two horses seroconverted to H7N7 by HI during the outbreak. One of these horses also seroconverted by SRH.

Using the ID Screen Influenza A H7 Antibody Competition ELISA two suspects and one positive sample (3.23%) were identified on initial sampling. Six positives (19.35%) and two suspects were identified in convalescent samples. Three horses seroconverted to H7N7 by ID Screen Influenza H7 Antibody Competition ELISA during the outbreak. This included one horse that also seroconverted by SRH and two horses that seroconverted by ID Screen Influenza A H7 Antibody Competition ELISA only. The horse that seroconverted by both HI and SRH did not seroconvert by ID Screen Influenza A H7 Antibody Competition ELISA i.e. there was no horse that seroconverted by all three assays. Twenty seven horses did not seroconvert to H7N7 by SRH or ID Screen Influenza A H7 Antibody Competition ELISA, however, one of these horses seroconverted by HI (Table 4.13).

**Table 4. 13 Percentage of seroconversions to H7N7 post natural infection with an H3N8 virus detected by the three tests**

| Category | Seroconversions detected | Number of Horses (%) |
|---|---|---|
| 1 | SRH and H7 ELISA | 1/31 (3.23%) |
| 2 | SRH and HI | 1/31 (3.23%) |
| 3 | H7 ELISA only | 2/31 (6.46%) |
| 4 | HI Only | 1/31 (3.23%) |
| 5 | Negative by SRH, HI and ELISA | 26/31 (83.87%) |

**H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis, HI= Haemagglutination Inhibition**

## 4.3.1.1 Statistical Analysis of H7N7 Diagnostic Tests Post Natural Infection

**Table 4. 14 Cohen's kappa scores for diagnostic methods for H7N7 post natural infection**

| Test 1 | Test 2 | Kappa Scores | SE of Kappa | 95% Confidence Interval | Strength of Agreement |
|--------|--------|--------------|-------------|-------------------------|-----------------------|
| SRH | H7 ELISA | 0.484 | 0.154 | 0.183 to 0.786 | Moderate |
| SRH | HI | 0.156 | 0.149 | -0.146 to 0.448 | Slight |
| H7 ELISA | HI | -0.104 | 0.032 | -0.166 to -0.042 | Slight |

**H7 ELISA= ID Screen Influenza H7 ELISA, SRH=Single Radial Haemolysis**

The agreement between tests for H7N7 post natural infection ranges from 'moderate' to 'slight' (Table 4.14). The acute samples or convalescent samples had varying results between tests with the SRH and ID Screen Influenza H7 Antibody Competition ELISA having the most similarities and the ID Screen Influenza H7 Antibody Competition ELISA and HI having the least.

### 4.3.2 Detection of H7N7 in Samples Post Vaccination

The samples from the 63 weanlings that had participated in the comparative vaccine study were tested using the ID Screen Influenza A H7 Antibody Competition ELISA and the results compared to those previously obtained by SRH (Gildea et al., 2011c). The antibody response to H7N7 following vaccination is illustrated in Figure 4.15. Weanlings with MDAs against H7N7 were excluded from the analysis.
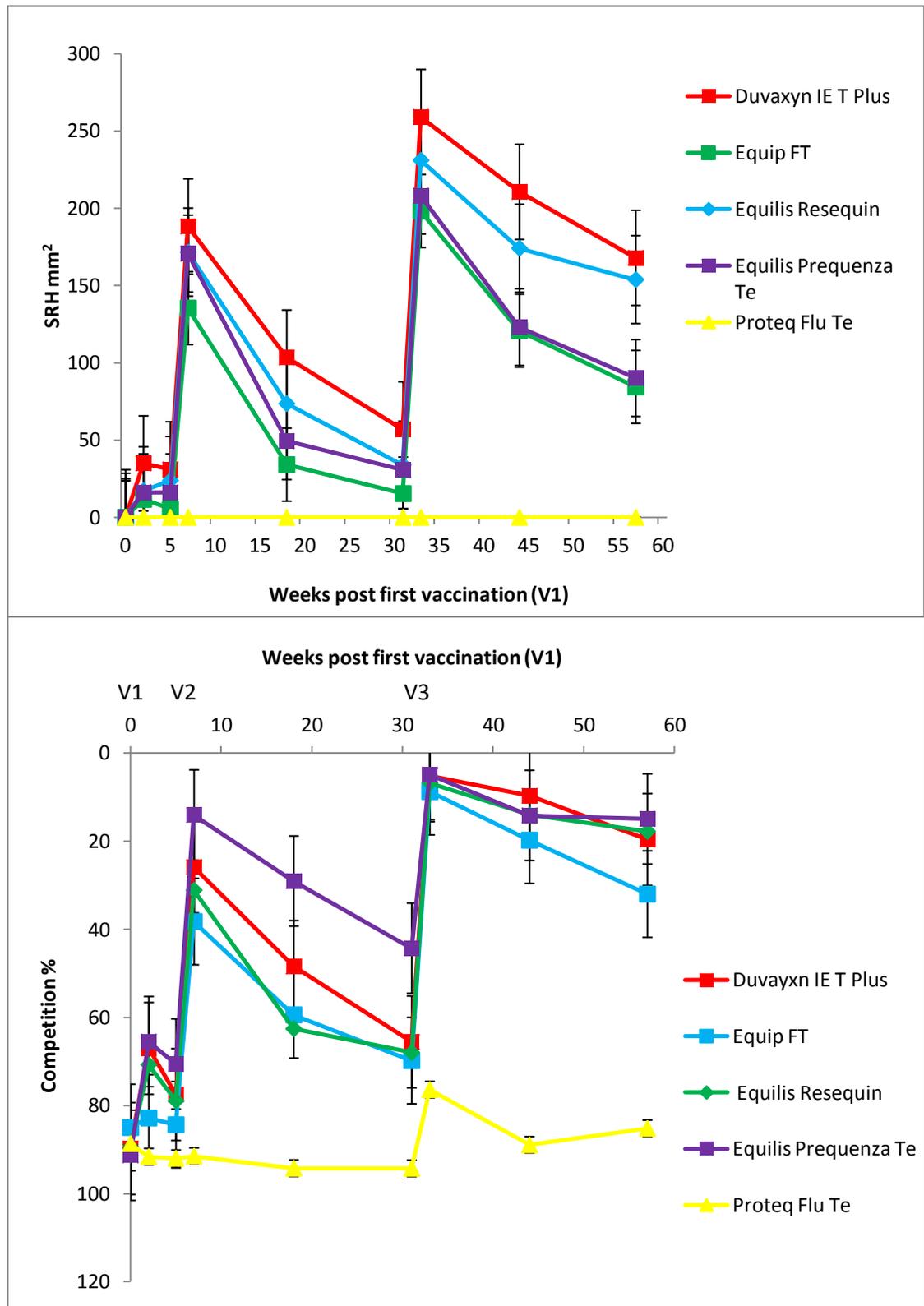
**Figure 4. 15 Mean H7 antibody level as determined by SRH or H7 ELISA post vaccination (n=63)**

The pattern of antibody response detected post vaccination with the ID Screen Influenza A H7 Antibody Competition ELISA was similar to that detected by the SRH in this study (Figure 4.15).

A comparison between the patterns of antibody response (mean competition percentage) obtained using the ID Screen Influenza A Antibody Competition ELISA and the ID Screen Influenza A H7 Antibody Competition ELISA is illustrated in Figure 4.16.
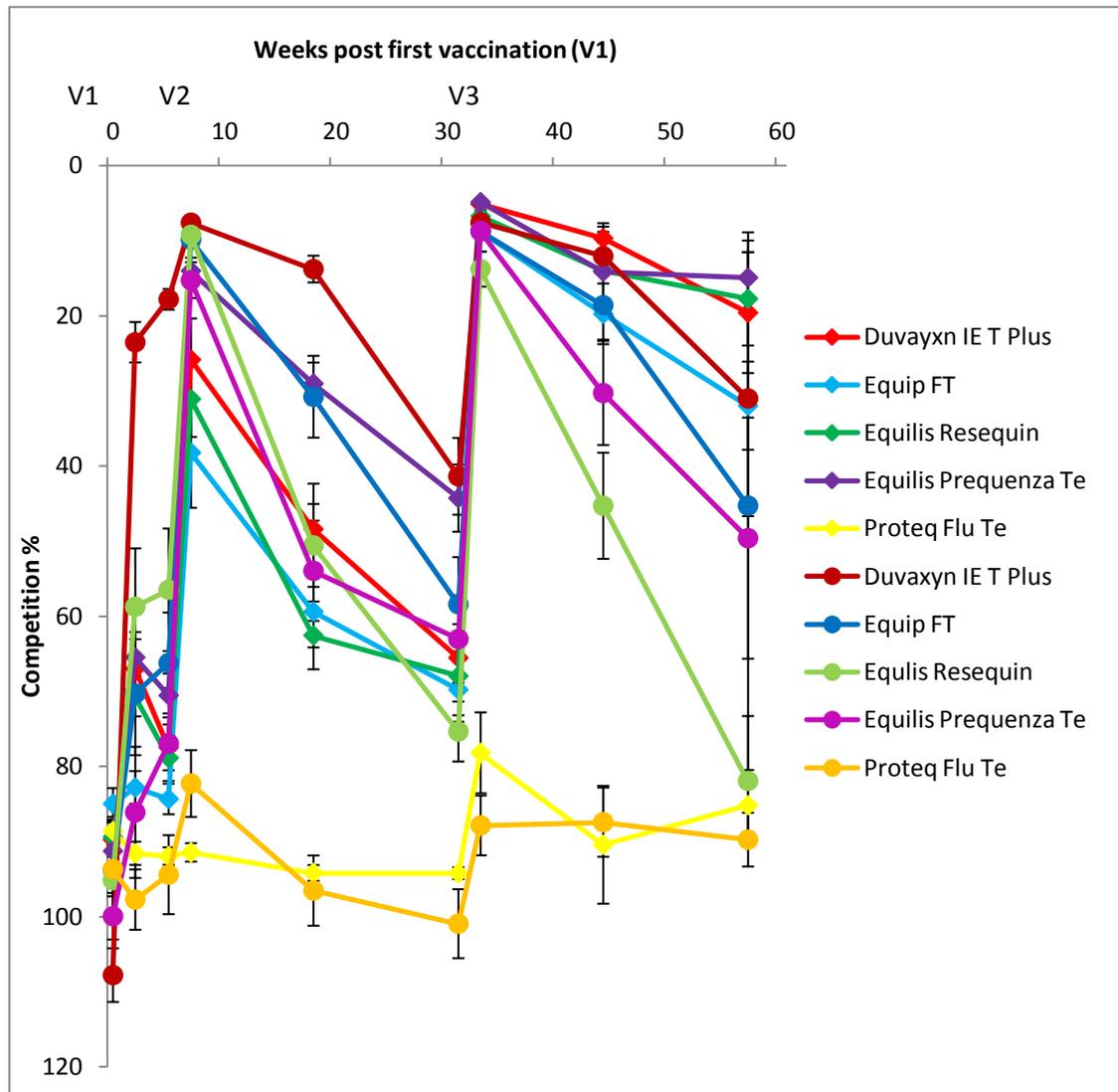


**Figure 4. 16 Mean H7 competition percentage as determined by Antibody Competition ELISA and H7 ELISA post vaccination**

◯ = **Antibody ELISA**   ◇ = **H7 ELISA**

**H7N7 Antibody Responses to primary vaccination**

With the exception of one horse vaccinated with Equilis Resequin, none of the horses included in this study had mounted an ID Screen Influenza A H7 Antibody Competition ELISA response to H7N7 two weeks post V1. This was in contrast to the SRH results where 14-75% of horses tested positive in all vaccine groups (Figures 4.17-4.20). All of the horses mounted an ELISA response to H7N7 two weeks post V2 with the exception of three horses vaccinated with Equip FT, one horse vaccinated with Duvaxyn IE T Plus and one with Equilis Resequin. The horse vaccinated with Equilis Resequin and one of the horses vaccinated with Equip FT which failed to mount an ELISA response to H7N7 also failed to mount a H7N7 antibody response by SRH. Two further horses vaccinated with Equip FT and one with Equilis Prequenza failed to mount a H7N7 antibody response two weeks post V2 by SRH only.

A decrease in the number of seropositive weanlings was observed using both assays three months post V2 and at the time of V3. The greatest reduction in seropositives was observed using the ID Screen Influenza H7 Antibody Competition ELISA in horses vaccinated with Duvaxyn IE T Plus, Equilis Resequin and Equip FT. This was in contrast with the greatest reduction in seropositives observed using the SRH test in horses vaccinated with Equilis Prequenza. Two weeks post V3 all horses were identified as seropositive using both assays. Three months and six months post V3 an equal number of seropositives were identified in all vaccine groups using both assays with the exception of horses vaccinated with Equilis Prequenza Te when at six months post V3 a higher percentage of seropositives were identified using the ID Screen Influenza H7 ELISA Antibody Competition ELISA (100%) in comparison to the SRH test (75%). Proteq Flu Te did not induce an antibody response as measured either by SRH or ID Screen Influenza H7 Antibody Competition ELISA. The mean competition percentage of the negative samples can be seen in Figure 4.21.
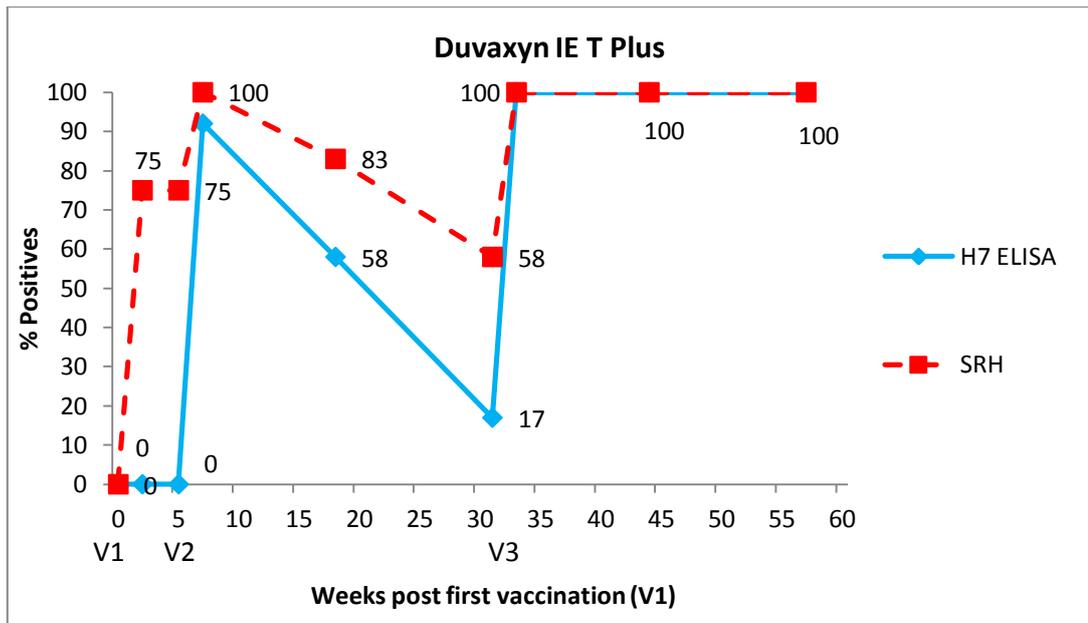
**Figure 4. 17 Percentage of seropositive (H7N7) weanlings as determined by H7 ELISA or SRH, following vaccination with Duvaxyn IE T Plus**

H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis
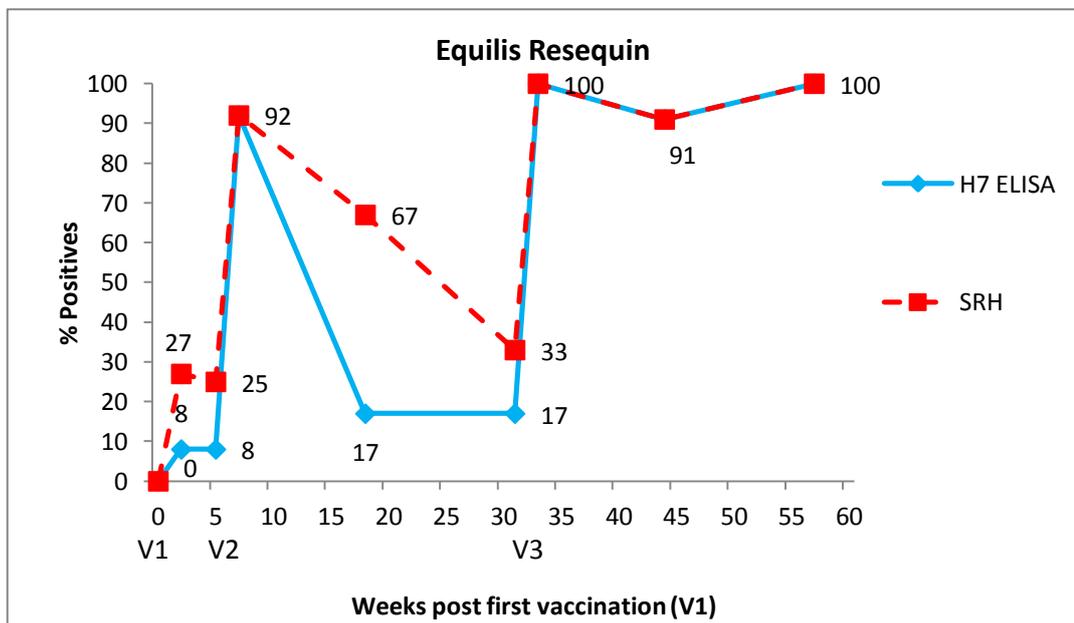


**Figure 4. 18 Percentage of seropositive (H7N7) weanlings as determined by H7 ELISA or SRH, following vaccination with Equilis Resequin**

H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis

64
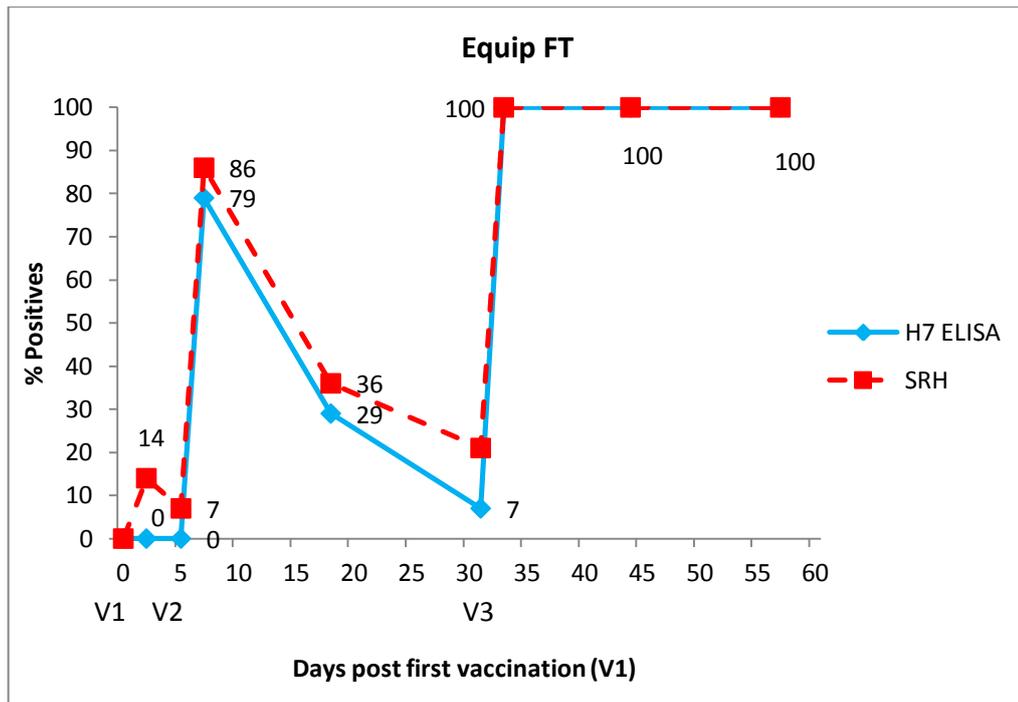
**Figure 4. 19 Percentage of seropositive (H7N7) weanlings as determined by H7 ELISA or SRH, following vaccination with Equip FT**

H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis
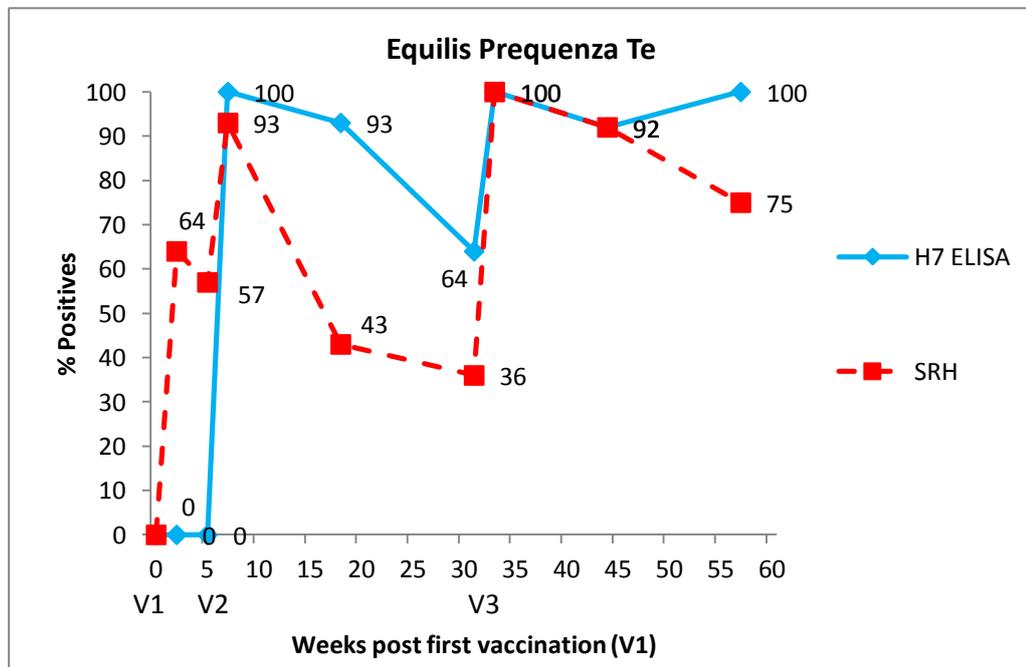


**Figure 4. 20 Percentage of seropositive (H7N7) weanlings as determined by H7 ELISA or SRH, following vaccination with Equilis Prequenza Te**

H7 ELISA= ID Screen Influenza H7 Antibody Competition ELISA, SRH=Single Radial Haemolysis

65
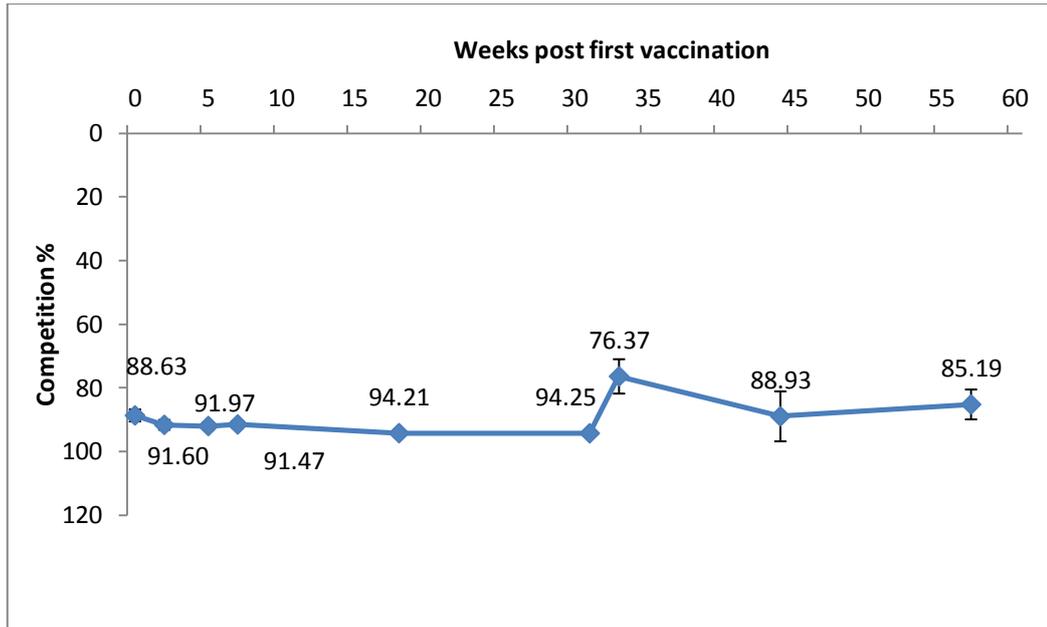
**Figure 4. 21 Mean competition percentage of negative (H7N7) weanlings as determined by H7 ELISA, following vaccination with Proteq Flu Te**

**4.3.2.1 Statistical Analysis of Diagnostic Tests for Samples Post Vaccination with H7N7**

The comparison between the SRH and the ID Screen Influenza H7 Antibody Competition ELISA shows that the agreement between the assays was 'substantial'. The kappa score was 0.687 (CI 0.626 to 0.749, SE $\pm$ 0.032).

# 5   *Discussion*

The rapid and accurate detection of equine influenza is essential if the laboratory diagnosis is to have a significant impact on the management of disease. Sensitive, specific and rapid tests are necessary to ensure the isolation of infected horses and the prevention of transmission to susceptible horses, to prevent unnecessary treatment with antibiotics and to encourage vaccination in the wider population. These tests are also essential to monitor the status of vaccinated horses in quarantine both prior to export (PEQ) and post arrival (PAQ) to prevent the introduction of virus to susceptible populations. This study compared the sensitivity of two RAD kits and a commercially available ELISA to VI and RT-PCR.

The results of the comparative studies in both naturally infected and experimentally challenged horses confirmed that RT-PCR is the most sensitive technique available for the detection of EI. This was consistent with previous studies (Quinlivan et al., 2004; 2005; van Maanen et al., 2003; Read et al., 2012). Testing of the clinical samples indicated that RT-PCR is useful for the screening of sub-clinically infected and vaccinated horses. These findings were similar to those of Gildea et al. (2011a) in an investigation of outbreaks of EI on premises with mixed vaccination history. In the challenge study a SYBR Green based RT-PCR assay was used and there was 'substantial' agreement between RT-PCR and VI. Positive samples were detected up to 10 days post challenge by RT-PCR compared to up to seven days post challenge by VI. However, positive results with the more sensitive RT-PCR assays such as the probe based assay used in this study to test the clinical samples; do not always correlate with the presence of replicating virus (Read et al., 2012). The examination of samples from outbreaks of EI in this study identified 11 horses that were positive by RT-PCR that tested negative by all other virus detection tests. Paired sera were available for seven of these horses and none of them seroconverted. During the 2007 outbreak in Australia it was demonstrated that horses may test positive by RT-PCR long after there is a likelihood they are infectious and constitute a risk to other horses (Read et al., 2012). RNA was detected up to 34

days after infection although experimental challenge studies estimate that horses remain infectious for less than 14 days. Thus other test results along with clinical and epidemiological data need to be considered when interpreting positive RT-PCR tests.

The RADs and the ID Screen Influenza A Antigen Capture ELISA are similar to RT-PCR in that they also detect a viral component rather than viable virus. Directigen Flu A, Espline Influenza A&B-N and the ID Screen Influenza A Antigen Capture ELISA test are all based on the binding of influenza A viral NP to antibody that is specific for this highly conserved protein. Directigen Flu A and Espline Influenza A&B-N have both been shown previously to effectively detect non-human influenza A viruses (Ryan-Poirier et al., 1992; Bai et al., 2005) and in a comparative study have been shown to be the most sensitive RADs for the detection of EI (Yamanaka et al., 2008). The ID Screen Influenza A Antigen Capture ELISA is marketed as a diagnostic test for swine, equine and avian influenza. In this study, Directigen Flu A proved to be the most sensitive of the three tests in the examination of clinical and experimental samples from horses exposed to EI. Espline Influenza A&B-N was slightly more sensitive than the ID Screen Influenza A Antigen Capture ELISA for the detection of EI post challenge but this was reversed when testing clinical samples. The superiority of Directigen Flu A was confirmed by comparing the limit of sensitivity of the three assays using known concentrations of virus. The Directigen Flu A was able to detect less than 1HA unit of virus, a result consistent with that reported by Chambers et al. (1994). Espline Influenza A&B-N and the ID Screen Influenza A Antigen Capture ELISA had a limit for detection of 5.5 HA units of virus. The findings differ from those of Yamanaka et al. (2008) who found similar detection limits for Espline Influenza A&B-N and Directigen Flu A in virus stock and almost equal sensitivities in the detection of virus in nasal swabs from three experimentally infected horses. The samples used by Yamanaka et al. (2008) were similar to those in this study in that they were frozen prior to testing. However the horses were older (two years old as opposed to foals) and the challenge dose was greater ($10^{8.6}$ EID/ml compared to $10^6$ EID/ml). It is possible that these differences particularly the higher challenge dose, may have impacted on the results. Yamanaka et al.

(2008) also reported two apparent false positive results with Directigen Flu A and suggested that the specificity of Espline Influenza A&B-N was superior to Directigen Flu A. The specificity of Espline Influenza A&B-N and of the ID Screen Influenza A Antigen Capture ELISA was not called into question in this study as all samples detected as positive by one or both of these tests also tested positive by other assays. However, in the experimental study presented here three weak positive samples were detected by Directigen Flu A on days 11, 13 and 14 post challenge. The time post challenge and the fact that these positives were not detected by any other diagnostic method suggest that the veracity of the results is open to question. Waner et al. (1991) reported false positive results with Directigen Flu A in human samples and advised that weak Directigen Flu A positives may sometimes be due to non-specific reactions which can be reduced by freezing the samples prior to testing. The samples in this study were mostly frozen prior to testing but as with RT-PCR results it may be best to interpret weak Directigen Flu A positive results in conjunction with other data. No cross reactivity of Directigen Flu A with a panel of other equine respiratory viruses was observed (Table 4.5) but further studies need to be carried out with other microorganisms.

In the study presented here, the Directigen Flu A test exhibited very different sensitivity compared to VI in eggs in the analysis of field samples compared to the results obtained using samples from experimentally challenged foals. The percentage of clinical samples detected as positive by Directigen Flu A was over 30% compared to less than 3% by VI. In contrast, the percentage of samples from experimentally infected horses detected as positive by Directigen Flu A was 16% compared to 36% by VI. This was consistent with previous studies by Chambers et al. (1994), Quinlivan et al. (2004) and Yamanaka et al. (2008) who reported that Directigen Flu A was less sensitive than VI for the detection of virus in experimentally challenged horses. It has been suggested that Directigen Flu A is most useful at the peak of infection but less sensitive early or late in infection when low levels of virus are shed (Mumford et al., 1995). In the experimental challenge study presented here, peak viral shedding occurred from Day 2 to Day 6 post challenge and this was the period when the majority of positive swabs identified by Directigen Flu A were collected.

However, the samples with the highest viral titre were not always detected by Directigen Flu A. The nature of the sample influences the sensitivity of the assay as well as the quantity of virus present. Ryan-Poirier et al. (1992) found that Directigen Flu A detects cell-associated antigens more readily than free virus. They detected swine influenza in lung homogenates and avian influenza in faecal material despite the presence of large amounts of particulate matter and suggested that an increase in cellular material in the specimen may increase the sensitivity. In humans, the Directigen Flu A has been shown to be more effective for nasopharyngeal aspirates than for swabs or gargles (Ryan-Poirier et al., 1992; Kaiser et al., 1999; Landry and Ferguson, 2003). In the study presented here all nasal secretions were centrifuged prior to testing. Centrifugation is not recommended by the manufacturer of Directigen Flu A and may have affected the sensitivity of the test. In the experimentally challenged foals, the greater sensitivity of VI over Directigen Flu A and the other antigen detection tests may have been related to the virus strain used to challenge the foals. Some virus strains are more readily isolated and propagated in eggs than others. The challenge virus used in this study A/eq/Kildare/89, a virus of the European lineage, was far easier to isolate in eggs than the viruses of the American lineage that have been responsible for the majority of the outbreaks in recent years (OIE, 1996; Chambers et al., 1994).

The published data concerning the ability of Directigen Flu A to detect EI in clinical samples are similar to those related to the diagnosis of human influenza in that different sensitivities have been reported. Evaluation of the assay in human hospitals demonstrated a wide range of sensitivities for example, 100% (Waner et al., 1991), 62% (Johnston and Bloy, 1993) and 61% to 77% (Newton et al., 2002) when compared to virus culture. In a retrospective study of frozen samples from an EI outbreak in Hong Kong, the Directigen Flu A was 84% as sensitive as VI and only 58% as sensitive when evaluating fresh samples from suspected field infections in the USA (Chambers et al., 1994). In the investigation of 23 outbreaks of respiratory disease in Ontario, Directigen Flu A proved less sensitive than VI (Diaz-Mendez et al., 2010). In contrast, there are several studies that report Directigen Flu A as more sensitive than VI for the evaluation of clinical samples from horses. Morley et al. (1995b) found that

between 33% and 45% of the nasal secretions collected from horses during influenza epidemics were positive by Directigen Flu A compared to only 7% by VI. In the investigation of the first EI outbreak to be reported in Dubai, virus was only isolated from two of the 17 horses that were positive by Directigen Flu A (Wernery et al., 1998). In an investigation of an outbreak of influenza in the Netherlands five of 11 nasal swabs were positive by VI, seven were positive by the Directigen Flu A and nine were positive by PCR (van Maanen et al., 2003). Quinlivan et al. (2005) examined 171 nasopharyngeal swabs from horses with respiratory disease and reported that virus was isolated in eight, detected by Directigen Flu A in 14, and by RT-PCR in 35 swabs. In the retrospective examination of clinical samples in this study only two samples were positive by VI compared to 23 by Directigen Flu A. The samples were mostly frozen prior to testing and the freeze-thaw may have decreased the infectivity of virus (Quinlivan et al., 2004). The Directigen Flu A does not require virus to be viable for detection. However, it is more likely that a delay in requesting veterinary assistance on premises affected by EI led to the period of peak virus shedding being missed in sampling. An investigation of outbreaks of EI in Ireland revealed that veterinary advice was sought on average more than five days after the first clinical signs were observed (Gildea et al., 2011a).

RT-PCR is the most sensitive test for the timely diagnosis of EI but a sensitive RAD may be useful in certain circumstances as it can be performed outside a laboratory and provide a rapid indication of the presence of virus. Directigen Flu A and Espline Influenza A&B-N take approximately 15 minutes, require no specialised equipment and can be performed by personnel that are not specially trained in virological techniques. In this study, Directigen Flu A was found to be more sensitive than Espline Influenza A&B-N. Directigen Flu A was also shown to be more sensitive than the laboratory based ID Screen Influenza A Antigen Capture ELISA and simpler and more rapid to perform. The ID Screen Influenza A Antigen Capture ELISA takes approximately two hours and is suitable for high throughput testing. However, this study found a low rate of positive detection with this test suggesting that it is insufficiently sensitive to accept negative results. RT-PCR has also been viewed as the most sensitive test, based on its ability to correctly identify horses with the EI virus. This test also

appears to be one of the most specific, based on the low proportion of false positives. In the present series of experiments, RT-PCR was also the most sensitive diagnostic method for identifying EI virus following post natural or post challenge infection samples.

The detection of virus shedding in subclinically infected horses is essential to prevent the introduction of virus to susceptible populations resulting in explosive spread of influenza with serious economic losses. This study illustrated that Directigen Flu A can detect virus in some subclinically infected and vaccinated horses indicating that if RT-PCR is unavailable, it could be used to screen horses in quarantine. Directigen Flu A is used routinely to screen imported horses in quarantine in Dubai. In 2012, infected endurance horses imported from Uruguay into The Dubai Racing Club Quarantine tested positive by Directigen Flu A and the virus was subsequently isolated and characterized. RADs are also used to screen imported horses to Hong Kong, a practice introduced after the outbreak of EI in 1992 (Powell et al., 1995; Powell, 2008). Swabs are collected in quarantine on the first working day post arrival and prior to release. They are also taken from any horses showing signs of fever or respiratory disease. Recently the Hong Kong Jockey Club has replaced Directigen Flu A with the Espline Influenza A&B-N kit as it is a 2-step test with no additional reagents required. Although Espline Influenza A&B-N is easier to use, this study suggests that Directigen Flu A is more sensitive and thus has superior potential for preventing an incursion of EI.

Antibodies against EI are traditionally quantified by HI or SRH. Both assays measure antibodies against haemagglutinin and thus the titres correlate with protection. Neither test requires costly equipment but both need to be performed and interpreted by scientifically trained personnel. HI is frequently the serological test of choice for diagnosis as the results can be obtained within hours. The SRH test is more reproducible between laboratories but is more complicated and time consuming than HI and usually only performed in specialist laboratories. During the Australian outbreak in 2007, virus spread was limited by restriction of horse movement and strategic vaccination. The vaccine used in the eradication programme was Proteq Flu Te, a canary pox

recombinant vaccine that expresses only the haemagglutinin gene of EI. One of the reasons for choosing this vaccine was that it was possible to differentiate between vaccinated and infected horses (DIVA) using an ELISA that detected antibodies against the viral NP (Garner et al., 2011; Kirkland and Delbridge, 2011). The ELISA proved to be extremely useful in the control and eradication of EI in Australia (Sergeant et al., 2009; Read et al., 2012).

This study evaluated the sensitivity of a NP ELISA in naturally infected horses, experimentally infected horses and horses vaccinated with whole inactivated vaccines, subunit vaccines and a canary pox recombinant vaccine. The examination of paired serum samples from 203 horses on 14 premises affected by EI indicated that the ID Screen Influenza A Antibody Competition ELISA detected only 50 seroconversions compared to over 80 by HI or SRH. Many of the horses that seroconverted by the traditional tests but failed to seroconvert by ID Screen Influenza A Antibody Competition ELISA were seropositive by ELISA at the time the first or acute sample was collected. The results suggest that the ID Screen Influenza A Antibody Competition ELISA is a less sensitive test than HI or SRH for the detection of EI in a country where the virus is endemic and many of the horses are seropositive by ELISA at the time of exposure. In such circumstances the ID Screen Influenza A Antibody Competition ELISA should only be the test of choice in laboratories that do not have the expertise to perform HI or SRH. However analysis of samples from experimentally infected foals confirmed the findings of Read et al. (2012) that in the naïve animal a NP ELISA is extremely sensitive and can detect an antibody response by day seven post infection (Figure 4.5, 4.6). This sensitivity combined with the fact that the ELISA unlike the HI or the SRH, can be readily automated and used to rapidly screen large numbers of samples suggest that it is an appropriate test for monitoring the spread of EI in a naïve population. A NP ELISA was used to screen approximately 62,000 samples in six months after EI was detected in Australia in 2007 (Read et al., 2012).

In this study serum samples from weanlings that had participated in a comparative vaccine study were tested by ID Screen Influenza A Antibody Competition ELISA and the results compared to those previously obtained by

SRH (Gildea et al., 2011c). Only the antibody response of those vaccinated with Proteq Flu Te was not detected by ID Screen Influenza A Antibody Competition ELISA. Thus, this technique could not be used to differentiate infected horses from those vaccinated with the subunit vaccines Equip FT and Equilis Prequenza Te or the whole inactivated vaccines Duvaxyn IE T Plus and Equilis Resequin. A combination of this kit and any of these four vaccines would not have been suitable for the eradication programme in Australia as there is no DIVA capacity and it would not be possible to prove freedom from infection. This was to be expected for the whole virus inactivated vaccines but some DIVA capacity might have been anticipated with the subunit vaccines.

The pattern of antibody response as measured by ID Screen Influenza A Antibody Competition ELISA post vaccination was similar to that of the SRH for the four vaccines and the decline in antibodies after the second vaccination (V2) and prior to third vaccination (V3) was evident. The ID Screen Influenza A Antibody Competition ELISA was similar to the SRH in detecting a greater antibody response in horses vaccinated with Duvaxyn IE T Plus than other vaccines. The ID Screen Influenza A Antibody Competition ELISA was also similar to SRH in that no poor responders to the first dose (V1) of Duvaxyn IE T Plus were identified. The numbers of poor responders to V1 of Equilis Resequin and Equip FT were similar to those identified by SRH but the number of weanlings that failed to seroconvert to V1 following vaccination with Equilis Prequenza Te far exceeded those indentified by SRH. A possible explanation for this finding is that Equilis Prequenza Te is an ISCOM matrix based subunit vaccine and may contain less NP than haemagglutinin. As with SRH, almost all of the weanlings that failed to seroconvert to V1 seroconverted to V2 by ID Screen Influenza A Antibody Competition ELISA. The high incidence of poor responders amongst these Thoroughbred weanlings reported by Gildea et al. (2011c) has not been reported previously in experimental studies with these vaccines. This study using a different serological test corroborates the original findings of Gildea et al. (2011c). Poor responders are a source of concern to horse owners and trainers. They are considered to be most likely to be the index cases in the event of an outbreak in a vaccinated population (Wood, 1991).

The interference of MDAs with the response to EI vaccines that is observed by SRH was also evident by ID Screen Influenza A Antibody Competition ELISA although only in the case of one horse. In this study one weanling was seropositive by ID Screen Influenza A Antibody Competition ELISA at the time of V1 and it failed to respond but seroconverted by ID Screen Influenza A Antibody Competition ELISA to subsequent vaccinations.

Samples from a second comparative vaccine study in racehorses following booster vaccination (Gildea et al., 2011b) were analysed by ID Screen Influenza A Antibody Competition ELISA. The DIVA capacity of the ID Screen Influenza A Antibody Competition ELISA combined with Proteq Flu Te was again evident as no antibody response was detected post vaccination. The ID Screen Influenza A Antibody Competition ELISA response to all other vaccines used was similar to the SRH response, an initial peak followed by a decline within three months. However, in several horses the ID Screen Influenza A Antibody Competition ELISA determined response to booster vaccination was less persistent than that measured by the SRH. Read et al. (2012) reported that the ID Screen Influenza A Antibody Competition ELISA only detected 50% of horses 12 months following natural infection. The ID Screen Influenza A Antibody Competition ELISA results in the present study corroborated the SRH data in that a large number (58%) of these horses did not seroconvert post vaccination. In many cases these were not the same horses as those that failed to seroconvert by SRH but for both assays the antibody response correlated inversely with the level of antibody at the time of vaccination.

Although H7N7 co-circulated with H3N8 viruses in horses for many years the H7N7 viruses have not been isolated for over three decades. They are considered extinct and the OIE no longer require that they be included in EI vaccines. However the majority of manufacturers persist with including an H7N7 virus in their vaccines. Paired samples from horses that seroconverted to H3N8 following natural infection were tested with the ID Screen Influenza H7 Antibody Competition ELISA. As with the SRH and HI tests, a small number of horses experienced an increase in reactivity without any history of recent exposure to H7N7 by vaccination. The possibility of a broad based anamnestic

response in a minority of previously vaccinated horses cannot be ruled out. This phenomenon requires further investigation.

The evaluation of the ID Screen Influenza H7 Antibody Competition ELISA using samples from the comparative vaccine study in weanlings demonstrated a good correlation with SRH results for all vaccines containing a H7N7 virus. The ID Screen Influenza H7 Antibody Competition ELISA response to H7N7 in these weanlings was more persistent than their ID Screen Influenza A Antibody Competition ELISA response to the NP. This ID Screen Influenza H7 Antibody Competition ELISA could be used to monitor vaccinal response to H7N7 viruses.

# 6    Conclusion

Although RT-PCR is the most sensitive test for the detection of EI, not all laboratories have the capacity to perform RT-PCR. This study evaluated the sensitivity of two RADs (Directigen Flu A and Espline Influenza A&B-N) and a commercial ELISA. Of the three, Directigen Flu A proved the most sensitive and as an RAD can be used as a "pen-side" test. It is rapid to perform and does not require scientifically trained personnel. Directigen Flu A is suitable for use in a quarantine facility if RT-PCR is not available as it may detect subclinically infected vaccinated horses. However results from the experimental challenge study suggest that the Directigen Flu A is most effective at the time of peak virus shedding. A negative Directigen Flu A result does not guarantee freedom from infection as a horse may be excreting low levels of infectious virus. Directigen Flu A is a valuable adjunct to laboratory techniques but negative samples from suspect cases should be submitted to a laboratory for further analysis by RT-PCR. Virus isolation should be carried out on all positive samples for strain characterization and surveillance.

Although a confirmatory serological diagnosis requires paired serum samples and thus is frequently retrospective, there is always a demand from clinicians for serological testing. The traditional or 'gold standard' serological assays for EI, SRH and HI require specialist expertise. In contrast, ELISA tests can be carried out in the majority of laboratories. The ID Screen Influenza A Antibody Competition ELISA detects antibodies against the internal NP of influenza viruses. The results of this study suggest that in an endemic population it is less sensitive than SRH or HI in detecting seroconversions but in a naïve population it may be more sensitive. As it is quicker to perform than HI or SRH and can be automated, it is suitable for mass screening of susceptible horses after an incursion of EI virus. Such an ELISA was found to be very valuable during the Australian outbreak when it had the additional benefit in its DIVA capacity to differentiate between naturally infected horses and those vaccinated with a canary pox recombinant expressing only the haemagglutinin. This study confirmed this DIVA capacity but demonstrated that it was restricted to the canary pox recombinant vaccine and did not pertain to two other subunit

vaccines tested. The ID Screen Influenza A Antibody Competition ELISA response to these ISCOM based subunit vaccines and to two whole inactivated virus vaccines tested was similar in pattern to the SRH response. Furthermore, the high incidence of poor responders amongst Thoroughbred weanlings to V1 and the failure of many older horses to seroconvert to booster vaccination as reported by Gildea et al. (2011b) were substantiated by the ID Screen Influenza A Antibody Competition ELISA results.

Unlike antibodies against haemagglutinin which are measured by HI and SRH, antibodies against the NP do not correlate with protection. Furthermore, individual horses' ELISA results did not necessarily correlate with their SRH levels. However, the results of these vaccine studies suggest that a laboratory that lacked the capacity to test by SRH could monitor trends in response to several vaccines both whole virus and subunit by ELISA. Similarly although H7N7 is thought to be extinct an ELISA that detects antibodies against these viruses which are contained in many vaccines has potential for measuring vaccinal response.

In summary the results of this study suggest that sensitive RADs and ELISAs may be useful supplementary tests in the diagnosis and management of EI and in the monitoring of vaccine performance.

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# *Appendix*

Table 4.11 Standard errors added

| Vaccine | V1 | S2 | V2 | S4 | S5 | V3 | S7 | S8 | S9* |
|---|---|---|---|---|---|---|---|---|---|
| **Duvaxyn IET Plus** | | | | | | | | | |
| MDA SRH (1) | 39.82 | 36.83 | 28.23 | 142.22 | 59.24 | 14.14 | 240.29 | 187.87 | N/A |
| MDA ELISA (1) | 29.81 | 33.98 | 42.78 | 4.34 | 12.28 | 50.54 | 4.34 | 9.87 | 13.58 |
| Poor responders SRH (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders ELISA (1) | 88.62 | 46.28 | 60.43 | 9.33 | 19.78 | 55.27 | 8.83 | 14.50 | N/A |
| Normal SRH (12) | 1.17 (SE±1.117) | 94.67 (SE±10.907) | 81.51 (SE±10.821) | 206.36 (SE±8.742) | 111.85 (SE±8.688) | 63.26 (SE±6.567) | 247.07 (SE±9.259) | 192.8 (SE±13.653) | 150.92 (SE±22.299) |
| Normal ELISA (11) | 107.81 (SE±3.566) | 21.48 (SE±1.882) | 17.82 (SE±1.384) | 7.62 (SE±0.375) | 13.80 (SE±1.776) | 41.40 (SE±5.108) | 7.60 (SE±0.484) | 12.08 (SE±1.797) | 31.81 (SE±15.684) |
| | | | | | | | | | |
| **Equilis Resequin** | | | | | | | | | |
| MDA SRH (2) | 53.94 (SE±23.632) | 37.26 (SE±23.765) | 32.84 (SE±26.095) | 32.83 (SE±28.255) | 0.00 (SE±0.000) | 0.00 (SE±0.000) | 128.35 (SE±6.148) | 67.05 (SE±1.847) | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (7) | 1.02(SE±1.017) | 10.62(SE±2.323) | 22.01(SE±12.855) | 108.50(SE±15.433) | 14.68(SE±7.036) | 5.45(SE±2.864) | 171.53(SE±12.640) | 111.33(SE±17.501) | 98.52(SE±25.288) |
| Poor responders ELISA (9) | 91.21(SE±5.968) | 77.80(SE±4.502) | 67.50(SE±6.350) | 9.79(SE±1.153) | 54.41(SE±6.121) | 79.10(SE±4.186) | 13.26(SE±2.656) | 43.63(SE±5.674) | 81.96 (SE±8.637) |
| Normal SRH (4) | 0.00 (SE±0.000) | 59.81 (SE24.326) | 45.57 (SE±31.402) | 165.61 (SE±19.161) | 69.06 (SE±21.317) | 44.37 (SE±14.897) | 185.93 (SE±12.659) | 125.75 (SE±8.285) | 74.07 (SE±24.462) |
| Normal ELISA (4) | 93.76 (SE±8.393) | 30.61 (SE±6.944) | 32.00 (SE±8.766) | 9.72 (SE±1.255) | 46.95 (SE±8.007) | 70.71 (SE±5.777) | 13.82 (SE±3.191) | 44.52 (SE±19.583) | |
| | | | | | | | | | |
| **Equip FT** | | | | | | | | | |
| MDA SRH (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (11) | 0.52 (SE±0.520) | 6.15 (SE±2.066) | 5.03 (SE±1.757) | 167.38 (SE±10.639) | 54.74 (SE±4.996) | 28.04 (SE±10.519) | 197.97 (SE±8.884) | 129.55 (SE±11.740) | 76.82 (SE±10.377) |
| Poor responders ELISA (10) | 92.08 (SE±3.182) | 83.87 (SE±7.879) | 76.70 (SE±6.747) | 11.85 (SE±1.834) | 39.45 (SE±7.039) | 65.42 (SE±7.173) | 9.07 (SE±0.838) | 23.96 (SE±6.451) | 45.29 (SE±36.395) |
| Normal SRH (3) | 0.00 (SE±0.000) | 119.07 (SE±11.956) | 67.48 (SE±11.532) | 215.23 (SE±14.858) | 103.14 (SE±5.381) | 43.44 (SE±14.415) | 193.14 (SE±8.204) | 123.23 (SE±7.983) | N/A |
| Normal ELISA (4) | 98.11 (SE±10.225) | 36.47 (SE±4.509) | 40.13 (SE±4.757) | 8.45 (SE±0.433) | 18.13 (SE±2.106) | 41.00 (SE±8.357) | 8.06 (SE±0.377) | 9.24 (SE±0.688) | N/A |
| | | | | | | | | | |
| **Equilis Prequenza Te** | | | | | | | | | |
| MDA SRH (1) | 53.53 | 55.77 | 43.29 | 128.53 | 41.11 | 20 | 165.96 | 116.15 | N/A |
| MDA ELISA (0) | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A | N/A |
| Poor responders SRH (1) | 0 | 6.07 | 5.44 | 123.8 | 6.09 | 5.67 | 89.45 | 16.64 | 6.52 |
| Poor responders ELISA (12) | 100.17 (SE±3.508) | 94.61 (SE±6.859) | 81.39 (SE±6.303) | 16.47 (SE±2.513) | 57.68 (SE±6.359) | 66.41 (SE±6.249) | 9.23 (SE±0.906) | 33.94 (SE±6.950) | 53.25 (SE±22.121) |
| Normal SRH (12) | 0.00 (SE±0.000) | 44.6 (SE±6.549) | 30.17 (SE±4.859) | 161.32 (SE±7.147) | 51.92 (SE±9.651) | 19.59 (SE±4.993) | 169.43 (SE±6.854) | 109.43 (SE±6.854) | 84.94 (SE±20.841) |
| Normal ELISA (2) | 88.80 (SE±3.487) | 37.08 (SE±2.986) | 45.60 (SE±24.245) | 13.08 (SE±8.059) | 41.69 (SE±27.751) | 53.69 (SE±12.288) | 5.38 (SE±0.643) | 9.22 (SE±0.035) | 38.79 (SE±0.000) |