Controlling equine influenza: traditional to next generation serological assays

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Abstract

Serological assays provide an indirect route for the recognition of infectious agents via the detection of antibodies against the infectious agent of interest within serum. Serological assays for equine influenza A virus can be applied for different purposes: diagnosing infections; subtyping isolates; surveillance of circulating strains; and to evaluate the efficacy of vaccines before they reach the market. Haemagglutination inhibition (HI) and single radial haemolysis (SRH) assays are most commonly used in the equine field. This review outlines how both these assays together with virus neutralization (VN) and ELISA are performed, interpreted and applied for the control of equine influenza, giving the limitations and advantages of each. The pseudotyped virus neutralization assay (PVNA) is also discussed as a promising prospect for the future of equine influenza virus serology.

Key Words

1. Equine Influenza

Equine influenza (EI) is a respiratory disease of equids that causes morbidity amongst unvaccinated and in some cases vaccinated horses worldwide. The equine influenza A virus, which belongs to the *Orthomyxoviridae* family, is enveloped and contains a single-stranded, eight-segmented RNA genome. Prophylaxis against EI is of great importance due to the economic burden of the disease. The global equine sporting industry has been detrimentally impacted by outbreaks of the disease since the late 1970s, and cancellation of race meetings still occur today. As well as participation in sport, equids remain a very valuable working animal in developing countries and thus outbreaks of EI in such areas are a concern (Virmani et al., 2010).

Transmission of EI is rapid, particularly amongst stabled horses; with large quantities of virus expelled during coughing episodes, EI is highly infectious. Naive equids typically present with clinical signs such as nasal discharge, coughing and pyrexia. Upon the onset of such signs, horses should be quarantined immediately in an attempt to prevent transmission to other individuals. Vaccinated horses with subclinical infections are problematic when trying to prevent the spread of disease. Therefore, when considering the regional, national or global transport of horses, quarantine before introduction to a new population is essential (Morley et al., 2000). Without effective quarantine, sub-clinically infected horses have the potential to cause devastating outbreaks such as that in Australia in 2007, which affected thousands of horses on a continent previously free from EI (Webster, 2011). Efforts by the Australian government to control the spread of the outbreak cost in excess of $1 billion (Callinan, 2008).

2. Control of equine influenza

2.1. Diagnosis

Diagnosis of an EI infection is usually achieved through identification of viral antigens or genetic material contained in nasal swab samples. However, the virus can be indirectly detected through serological assays that highlight the presence of antibodies to EI within an individual's serum. Comparisons between acute and convalescent samples (taken upon the onset of clinical signs and around 2 weeks later) are necessary to determine recent seroconversion. Serological diagnosis is a useful adjunct to directly testing for viral antigens or genetic material to confirm whether influenza is the cause of disease. Virus replication is transient and therefore the virus may be cleared by the time clinical signs are observed, particularly in animals that are partially protected by vaccination. Where vaccination is employed, serological assays can indicate possible cases of vaccine failure.

2.2. Vaccination

Vaccines are available for EI, and regulatory equine bodies including the BHA (British Horseracing Authority), British Show Jumping Association (BSJA) and FEI (Federation Equestrian International) impose a mandatory vaccination programme for horses competing on national and international circuits, respectively. However, as with other domesticated species such as cats and dogs, vaccination is only recommended and not required for the native and 'everyday' working horse.

Both cell-mediated and humoral immunity are necessary to combat influenza infection. Consequently, a desirable attribute of vaccines is the induction of cytotoxic T lymphocyte responses (CTL) as well as specific antibody responses (Slater and Hannant, 2000). However, cell-mediated responses do not prevent infection. When challenged with an influenza virus, neutralizing antibodies against the haemagglutinin (HA) protein on the virus

surface provide subtype-specific protection by inhibiting viral entry into host cells. Different approaches to producing effective vaccines are constantly under development, with serological assays of great importance in efficacy testing procedures (Paillot et al., 2006).

2.3. Surveillance

Surveillance of circulating EI virus strains is necessary to monitor the spread of disease, and to identify the most prevalent strains for inclusion in vaccines.

H7N7, the first equine influenza A virus to be isolated in 1956 in Prague, is presumed extinct as it has not been isolated for over 30 years and consequently is no longer recommended as a vaccine strain (Chambers, 2014). Equine H3N8 viruses were first identified in Miami 1963 and in the late 1980s diverged into Eurasian and American lineages (Daly et al., 1996). The American lineage has since diverged into South American, Kentucky and Florida sub-lineages with further divergence of the Florida sub-lineage into Clades 1 and 2 (Lai et al., 2001). The H3N8 subtype continues to circulate globally with very few nations (e.g. New Zealand and Iceland) that have not experienced EI (Cullinane and Newton, 2013).

Unless vaccines are periodically reviewed and updated, outbreaks amongst vaccinated populations are probable. The UK and Japan are two countries to have experienced outbreaks of EI in the last 15 years amongst vaccinated racehorses due to the use of an out-dated vaccine (Newton et al., 2006; Yamanaka et al., 2008). EI vaccines were introduced in the late 1960s, but a major outbreak in 1989 demonstrated the need for vaccine strains to be updated. Furthermore, after the divergence of the original single H3N8 lineage into American and Eurasian lineages, it was demonstrated that vaccines were more likely to be effective if they included a representative strain from both lineages (Daly et al., 2004; Yamanaka et al., 2014; Woodward et al., 2015). Ideally, vaccine strain efficacy would be determined through sequence data to avoid the need for pony challenge studies when deciding on vaccine updates

(Daly and Elton, 2013). However, the requirement to update vaccine strains is not simply dependent on the number of amino acid changes in the HA protein (Yamanaka et al., 2014; Woodward et al., 2015). Understanding which amino acid changes are antigenically critical is key to optimising protection.

At present in the UK, there is one vaccine available that meets the recommendations of the OIE (World Organisation for Animal Health), i.e. it contains two H3N8 strains, one each from the Florida sub-lineage Clades 1 and 2.

3. Current serological assays

3.1. Haemagglutination inhibition assay (HI)

(a) Principles of assay

Developed in the 1940s, the HI assay uses the agglutination effect of the HA protein binding sialic acid receptors on erythrocytes (red blood cells; RBCs) to identify the presence of antibodies within serum (Hirst, 1942). Serum must be appropriately pre-treated (i.e. with potassium periodate or receptor-destroying enzyme and heat) to remove any non-specific agglutinins. Ineffective pre-treatment of serum can lead to false positive results, as was demonstrated when kaolin was used when testing for antibodies against equine H7N7 (Boliar et al., 2006). A Tween 80/ether solution is typically added to the antigen, which increases HA activity for H3N8 subtypes and prevents viral infectivity and cross-contamination. However, although it increases the sensitivity of the assay, it can also reduce specificity (Mumford, 1992). A standard amount of antigen is added to each well of a 96-well plate and the reciprocal of the serum dilution at which agglutination is completely inhibited is reported (HI titre). A detailed protocol for the HI assay is available from OIE (OIE World Organisation of Animal Health, 2015). A minimum four-fold increase in titre from a paired sample of acute and convalescent serum is required for classification of seroconversion (Morley et al., 1995).

(b) Assay applications: Advantages and disadvantages

HI assays are widely used for HA subtyping, surveillance and vaccine testing. The seroprevalence of EI in Nigeria (Olusa and Adeyefa, 2009) and Israel (Aharonson-Raz et al., 2014) are recent examples of EI surveillance using HI. Furthermore, antigenic characterisation of new isolates is continuously carried out across Europe, North America and most recently Dubai with HI as the principle assay (Woodward et al., 2014). Isolates can be antigenically characterised by differences in HI titre when assayed against a panel of reference sera. Antiserum raised in ferrets produces more strain-specific antibodies than equine serum, and thus is best used for discriminating between different isolates (Mumford, 1992). Such data inform decision making on which strains should be included in current EI vaccines (Bryant et al., 2011).

HI is officially recommended by the OIE for vaccine testing. A vaccine against EI is considered satisfactory for inducing clinical protection if it stimulates a mean HI titre of ≥1:64; however it is also noted that the titre may need to be higher in order to confer protection against infection and viral shedding (European Medicines Agency, 2014b). A limiting feature of the HI assay is the focus on binding of the HA to sialic acid residues on RBCs. Antibodies that bind to the trimeric head of the HA and prevent haemagglutination are not necessarily representative of protective neutralizing antibodies; thus HI titres do not necessarily correlate with protection. Nevertheless one report has suggested that the HI assay shows good correlation with a virus neutralization assay for EI (Morley et al., 1995).

The HI assay is not standardized and inter-laboratory variability of the assay is becoming a well-documented issue in influenza serology. The same serum panel was tested against EI across eight laboratories and up to a four-fold difference in HI titres was observed (Daly et al., 2007). Natural variation between different species and individual's RBCs as well

as treating virus with Tween 80/ether solution, increases assay variability. Furthermore, interpreting a titre from an HI plate is subjective. Despite the limitations discussed, the simplicity and relatively small associated costs mean that the HI assay is often favoured for both diagnosis and surveillance, particularly in resource-poor countries.

3.2. Single radial haemolysis (SRH)

(a) Principles of assay

Since the late 1970s, SRH has been used to detect antibodies against influenza (Schild et al., 1975). The SRH assay exploits the cell lysis properties of complement, in the presence of antibodies within a serum sample, to create a zone of haemolysis within an agar plate that contains virus-coated RBCs. Complement factors bind to anti-HA antibodies bound to the HA on the influenza virus treated RBCs. The size of the zone of lysis (usually measured in mm^2) correlates with the level of strain-specific antibodies that are present in the serum sample. The protocol provided by the OIE gives precise details on how to perform an SRH assay (OIE World Organisation of Animal Health, 2015). Seroconversion is defined by an increase in the zone of haemolysis by $25mm^2$ or 50%, whichever is smaller, between acute and convalescent serum samples.

(b) Assay applications: Advantages and disadvantages

In 2004, the OIE described SRH as the preferred assay for EI vaccine testing and although in 2008 it was only described as being of equal merit to the HI assay, it remains one of the only two 'trusted' serological assays to date (OIE World Organisation for Animal Health, 2004, 2008). The strong correlation between protective immunity post-vaccination and the detection of functional antibodies by SRH indicates why it remains a popular choice for testing vaccine efficacy. Correlates of protection have been defined for SRH by experimental

challenge of ponies. Individuals with antibody levels >150mm^2 are considered virologically protected against homologous vaccine-challenge strains, whereas higher levels are required where there is a mismatch between the vaccine strain and challenge strain. These protective antibody levels were supported by field studies of vaccine-induced antibody levels measured by SRH in young Thoroughbreds (Newton et al., 2000). Such cut-off values are used to inform the OIE on the necessity to update vaccines (Gildea et al., 2013).

SRH is more sensitive than HI because the serum is not diluted, thus providing a linear read-out, and hence an increase of 50% is required for confirmation of seroconversion rather than four-fold. Variation in SRH assays for EI has been documented with up to 3.9-fold differences across nine laboratories (Daly et al., 2007). The assay is, however, more reproducible than HI when reference sera are used (Mumford and Wood, 1993; Mumford et al., 1992). For diagnostic purposes, SRH is not often the assay of choice as it does not measure IgM antibodies and therefore may not detect early infection.

3.3. ELISA

(a) Principles of assay

An ELISA can be used to diagnose disease through the detection of specific antibodies that bind to a viral protein. There are variations of the assay such as blocking, indirect, competition and cell-based ELISAs, which highlight the flexibility of this assay platform. Typically for influenza diagnosis, the structural nucleoprotein (NP) is detected (Ji et al., 2011).

(b) Assay applications: Advantages and disadvantages

The ELISA is typically used for diagnosis due to its reasonable speed and high-throughput features. Screening procedures during the 2007 Australian EI outbreak, based on a blocking

ELISA, gave both sensitive and specific results. However, confirmatory testing by means of a different serological test was also necessary where unexpected results were observed (Sergeant et al., 2011). ELISA is not generally applicable for vaccine testing due to the semi-quantitative measure of antibody binding that is not necessarily representative of a neutralizing antibody response. The detection of anti-NP antibodies is also not subtype-specific, thus further testing is required to obtain more epidemiological information such as the subtype/strain. The ELISA is particularly useful for Differentiating Infected from Vaccinated Animals (DIVA), if a sub-unit vaccine has been used, because vaccinated individuals should not raise antibodies to all viral proteins if these are not included in the vaccine formulation (Galvin et al., 2013; Kirkland and Delbridge, 2011). A cell-based ELISA in which antibodies to the non-structural NS1 protein are measured has been suggested for use in equine DIVA because antibodies would only be expected to be present in infected equines not those vaccinated with inactivated virus (Rozek et al., 2011).

3.4. Virus neutralization (VN)

(a) Principles of assay

Virus neutralization assays identify antibodies capable of inhibiting virus entry and or replication within cells (Han and Marasco, 2011). Serum is heat-treated, serially diluted and incubated with a standardised amount of infectious virus before measuring a reduction in virus infectivity. The mixtures of serum and virus can be inoculated into the allantoic sacs of embryonated hens' eggs, the fluid harvested, and a VN titre assigned by measuring haemagglutination titres (Yamanaka et al., 2014), but this is quite a laborious assay. Quantification of neutralizing antibody responses by plaque reduction neutralization test (PRNT) in which an overlay (e.g. of agarose or carboxymethylcellulose) is added to prevent the virus from spreading across the plate in the supernatant is generally regarded as a gold

standard measure of VN antibodies. However, although MDCK cells supplemented with trypsin are permissive to EIV (OIE World Organisation of Animal Health, 2015), many EIV strains produce only very limited, if any, cytopathic effects. As a consequence, VN antibodies are frequently measured by titration in a 96-well plate (micro-neutralization, MN) with various approaches taken to measure reduction of virus in the supernatant.

(b) Assay applications: Advantages and disadvantages

VN is sensitive and relatively specific for diagnosing infection, as it measures biological function. Nevertheless, due to the significant cost associated with such assays compared with HI, it is most applicable to measuring vaccine-induced responses. Furthermore, advice on necessary vaccine strain updates can be given based on VN results (Ozaki et al., 2001). It is important to note that VN can only be conducted using live virus and so necessary bio-containment protocols must be followed. VN is not traditionally regarded as a high-throughput or rapid assay, another reason it is not generally used in a diagnostic setting. However, by fixing cells in a MN plate then using NP ELISA to quantify virus replication, it is possible to attain results after 24 hours (Khurelbataar et al 2014).

4. Transition from Traditional Assays to 'Next-Generation' Assays

Established assays such as HI and SRH, used since the early 1930s and 1970s respectively, are still heavily relied upon today. Each has advantages for different applications: HI is quick to perform and is relatively cheap, properties that are useful for diagnosis; SRH measures functional antibodies and is more sensitive than HI, which promotes its use in vaccine efficacy testing. A point to consider is that both use whole virus, and therefore cannot readily distinguish between vaccinated individuals and natural infections

(Young and Lunn, 2000). An outstanding issue is that neither HI nor SRH are standardised and both demonstrate high levels of inter-laboratory variability (Wood et al., 2011). For efficacy testing of human influenza vaccines, it has been suggested that, where possible, a centralised laboratory should perform all assays necessary to complete a development programme (European Medicines Agency, 2014a). Although the VN assay is more variable than the HI assay, is difficult to reproduce, and has high cost implications, its ability to quantify neutralizing antibodies is very useful (Stephenson et al., 2007). The measurement of virus neutralizing antibodies is the gold standard for evaluating vaccine efficacy. The problem is that VN assays are difficult to perform for EIV due to the lack of cytopathic effect. An alternative assay that overcomes the issues related to VN, still with a focus on measuring virus neutralizing antibodies, would provide an optimistic future for improved vaccine evaluation.

4.1. Pseudotype virus neutralization assay (PVNA)

(a) Principles of assay

Pseudotypes were initially developed as gene therapy vectors but have since been employed for use in serological assays (Temperton et al., 2015). They are formed of a chimeric virion comprising the core of one virus while displaying the surface glycoprotein(s) of another. Gene deletions within the retroviral core render the virus replication-deficient. Only structural genes that enable the generation of virus particles, and a gene for reverse transcription and integration of a reporter gene into the target cell genome of choice, are used in PV production (Temperton et al., 2007). When using a luciferase reporter gene, luminescence acts as an indirect measure for virus infectivity. Once PVs are generated and taken forward into a PVNA, a knock-down in luminescence can be attributed to the presence of neutralizing antibodies that prevent the PV particles from entering the target cell line of choice. The

antibody titre that results in 50% neutralization (IC_{50}) is usually reported after 48 hours incubation at $37^{o}C$. Optimisation of influenza pseudotyped virus production is important to ensure efficient and high titre production of virus. A meta-analysis of influenza PV production has recently been conducted and a consensus protocol produced (Carnell et al., 2015).

(b) Assay applications: Advantages and disadvantages

The PVNA is a novel assay based on the principle of quantifying neutralizing antibody responses without the need for live virus or large quantities of serum (2─10µl per replicate). The PVNA provides a safe platform for viral assay work, particularly for highly pathogenic strains. Higher costs would not favour the PVNA for use in diagnostics and surveillance but it would be feasible and applicable for use in vaccine efficacy testing. Another benefit of the pseudotype platform is the ability to readily manipulate the HA, which can be useful for inserting mutations to mimic those that occur in nature (antigenic drift studies). PVs are more amenable than using reverse genetics to create strain variants. Here, the focus can be on one antigen with the aim of understanding the critical changes that are important for vaccine development.

Reproducibility of the PVNA needs to be fully addressed but so far the results of comparative tests between HI assays and the PVNA are in agreement for human influenza (Garcia and Lai, 2011; Yang et al., 2014). EI PVs have also been generated and employed in PVNAs. Thus far, it is thought that the assay demonstrates increased sensitivity as some serum samples were defined as negative by SRH but positive by PVNA. Overall 65% correlation was shown between the two assays (Scott et al., 2012).

Furthermore, a bivalent fluorescence-based microneutralization assay (BiFMA), which uses the PV platform enables two influenza isolates to be detected at one time using two different reporters (Baker et al., 2015). Similarly, multiplexing using different reporters

to detect different influenza subtypes is also possible (Molesti et al., 2014). Measuring antibodies against different isolates could be a great advantage for testing bivalent, and trivalent, EI vaccine responses.

5. Conclusions

Of the serological assays that are currently available, each has its own merits and drawbacks for different applications relevant to the control of equine influenza (summarised in Table 1). The key is choosing the appropriate assay for the application, together aiding the control of EI. ELISA and HI assays are beneficial for diagnostic purposes, whilst SRH and VN are more suited for vaccine efficacy testing. For surveillance, HI is most favourable. An optimistic future for vaccine efficacy testing may lie with the use of novel assays, such as the PVNA, which encompasses several important features; quantification of neutralizing antibodies in a high-throughput fashion, whilst avoiding the need for live virus or large quantities of serum.

Conflicts of interest statement

The authors declare that there are no conflicts of interest regarding the publication of this review.

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Table 1. Summary of assay applications. ✓ Possible application(s). ✓✓ Favourable

application(s) reflecting upon the discussed advantages of each assay.

SEROLOGICAL ASSAY	DIAGNOSIS	SURVEILLANCE	VACCINE TESTING
Hemagglutination Inhibition	✓✓	✓✓	✓
Single Radial Hemolysis	✓	✓	✓✓
ELISA	✓✓	✓	✓
Virus Neutralization	✓	✓	✓✓
PVNA	✓	✓	✓✓

Figure 1. Principles of the pseudotype virus neutralization assay. 1 – Target cells alone do not

luminesce. 2 – Virus added to target cells, the virus infects target cells and the reporter gene is

integrated into the cell genome causing cells to luminesce. 3 – Upon addition of an antibody typically

directed against the HA, the influenza pseudotyped virus is prevented from binding to the target cells

thus infection is reduced and the luminescence measured, decreases.