RESEARCH REPORT

The use of equine influenza pseudotypes for serological screening

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ABSTRACT

Standard assays used for influenza serology present certain practical issues, such as inter-laboratory variability,
complex protocols and the necessity for handling certain virus strains in high biological containment facilities.
In an attempt to address this, avian and human influenza HA pseudotyped retroviruses have been successfully
employed in antibody neutralization assays. In this study we generated an equine influenza pseudotyped lentivi-
rus for serological screening. This was achieved by co-transfection of HEK293T cells with plasmids expressing
the haemagglutinin (HA) protein of an H3N8 subtype equine influenza virus strain, HIV gag-pol and firefly lucif-
erase reporter genes and harvesting virus from supernatant. In order to produce infective pseudotype particles
it was necessary to additionally co-transfect a plasmid encoding the TMPRSS2 endoprotease to cleave the HA.
High titre pseudotype virus (PV) was then used in PV antibody neutralization assays (PVNAs) to successfully
distinguish between vaccinated and non-vaccinated equines. The sera were also screened by single radial hae-
molysis (SRH) assay. There was a 65% correlation between the results of the two assays, with the PVNA assay
appearing slightly more sensitive. Future work will extend the testing of the PVNA with a larger number of
serum samples to assess sensitivity/specificity, inter/intra-laboratory variability and to define a protective titre.

KEYWORDS: Influenza, pseudotype virus, viral screening, neutralizing antibody, H3N8, SRH, TMPRSS2

INTRODUCTION

Influenza infections in equine species are relatively com-
mon and can have significant economic impact. The primary
clinical manifestation is acute respiratory disease. To date,
infections have only been associated with two subtypes;
H3N8 and H7N7. There have been no isolations of H7N7
virus from horses for over 30 years (Webster, 1993), but
viruses of the H3N8 subtype continue to cause outbreaks
regularly across the globe, some affecting large numbers of
animals (reviewed in Daly et al, 2011).

Equine influenza infection induces neutralising antibodies
against the surface glycoproteins haemagglutinin (HA) and
neuraminidase (NA). This is the primary protective immune
response elicited by natural infection or vaccination, with cell-
mediated immunity playing a role in clearing virus-infected
cells following natural infection (Paillot et al, 2006). As for
human influenza, measurement of antibodies against the HA
in particular is important in studies to determine vaccine
efficacy (i.e., performance in experimental vaccination and
challenge studies) and effectiveness (i.e., how well a vaccine
performs when used routinely ‘in the field’).

The haemagglutination inhibition (HI) test is useful for virus
characterisation and diagnostic purposes as it is a strain-
specific assay. However, high levels of inter-laboratory vari-
ation are observed, as a result of which protective antibody
levels are ill-defined, ranging from titres of 8 to 128 (Mumford
and Wood, 1992). For equine influenza, the requirement to
treat the virus with Tween 80 and ether to improve the sensi-
tivity of the assay (John and Fulgitini, 1966) further increases
the sensitivity of the assay (John and Fulgitini, 1966) further increases
variability of the assay. The HI test only measures inhibition
of HA binding to cells (HA globular head reactive antibodies).
The single radial haemolysis (SRH) assay measures complement-mediated haemolysis induced by influenza antibody–antigen complexes. The SRH test can be standardised using reference sera (Daly et al., 2007) and a clear relationship has been demonstrated between pre-challenge antibody levels measured by SRH and protection afforded in challenge studies (Mumford and Wood, 1992). However, the SRH assay has disadvantages; it is relatively cumbersome to perform; requires the use of sheep blood and guinea pig complement; and resulting zones of haemolysis have to be measured either using specialised equipment or by hand using digital callipers.

Measurement of virus neutralising (VN) antibodies against equine influenza is rarely performed; VN assays in eggs are time-consuming, labour-intensive and the lack of cytopathic effect in cells often requires a two-step assay in which reduction in virus replication is measured by detection of viral protein by ELISA or other assays.

The use of equine influenza pseudotyped lentiviruses may provide a solution to the requirement for a reliable and reproducible assay to assess the level of neutralising antibodies to equine influenza in vaccinated or naturally-infected horses. A number of avian and human influenza virus subtypes are time-consuming, labour-intensive and the lack of cytopathic effect in cells often requires a two-step assay in which reduction in virus replication is measured by detection of viral protein by ELISA or other assays.

The aim of the current study was to develop a pseudotype virus neutralization assay (PVNA) for the measurement of neutralising antibodies to H3N8 subtype equine influenza viruses.

**MATERIALS AND METHODS**

**Viruses and sera**

Stocks of A/equine/Sussex/89 (H3N8) influenza virus (a European lineage strain; Daly et al., 1996) were grown in 10-day-old embryonated hens’ eggs. Serum samples surplus to diagnostic requirements were obtained from 20 routinely influenza-vaccinated donkeys. The donkeys were recently (within 80 days) vaccinated with a booster dose of a commercially available inactivated vaccine containing the American-lineage H3N8 strain A/equine/Newmarket/1/93, the European-lineage H3N8 strain A/equine/Newmarket/2/93 in addition to A/equine/Prague/56 (H7N7). The epitope-bearing HA1 regions of the Sussex/89 and Newmarket/93 viruses share 99% amino acid identity (as determined using CLUSTAL software). A batch of serum from a hyperimmunised experimental pony vaccinated with a number of H3N8 strains over two decades (A/equine/Suffolk/89, A/equine/Newmarket/1/93, A/equine/Newmarket/2/93, A/equine/Ohio/03), was used as a positive control. Serum samples from two horses, confirmed to be influenza-naïve by SRH, were kindly provided by Prof. Ann Cullinane, Irish Equine Centre. Sera were stored at -80°C until use.

**Generation of H3 pseudotyped virus**

Viral RNA was extracted from allantoic fluid using the QIAamp viral RNA extraction kit (Qiagen). The full haemagglutinin gene was amplified by Titan One Tube RT-PCR (Roche) using custom primers (Invitrogen) binding to the gene termini:

Forward: 5’-GCGCGCGGATCCGTCAAAATGAAGACATTTG
Reverse: 5’-GCGCGCTCGAGAATTTAAATGCAAATGGTTGCATCT

PCR fragments were then digested with BglII and XhoI restriction enzymes and cloned into the pl.18 expression plasmid as used previously for other influenza HA pseudotypes (Temperton et al., 2007). All plasmids were prepared and purified using kits from QiAGEN, according to the manufacturer’s instructions.

Influenza pseudotyped lentivirus particles expressing the firefly luciferase reporter gene were generated by plasmid co-transfection (Temperton et al., 2007; Figure 1). Approximately 4 × 10⁶ human embryonic kidney 293T/17 cells (American Type Cell Culture) were seeded into 10 cm diameter sterile, plastic dishes (Nunc) in DMEM medium with Glutamax and high glucose (Gibco, Invitrogen) plus 10% (v/v) foetal bovine serum (Sigma) and 1% (v/v) penicillin/streptomycin (Fisher) and placed at 37°C in a 5% CO₂ (v/v) humidified incubator for 24 hrs. Next, the pl.18-HA, gag/pol construct pCMV-D8.91 (Zufferey et al., 1997), firefly luciferase construct pCSFLW (Wright et al., 2008) and pCAGGS-TMPRSS2 protease plasmids (Bötcher et al., 2006) were combined and added to a Fugene6/OptiMEM medium mixture (Roche), and then to the cells according to the manufacturer’s instructions.

On the following day the medium was replaced and 1U of exogenous soluble Clostridium perfringens NA (Sigma) was added to facilitate sialic acid cleavage and pseudotype virus egress. Cell culture supernatant was harvested after further 24 hrs, passed through a 0.45 µm pore sterile syringe filter, aliquoted and stored at -80°C.

**Titration of pseudotyped virus**

Virus supernatant (5µl/well) was added to a 96-well plate along with 1 × 10⁴ HEK293T target cells and 200 µl total medium per well and incubated as above for 48 hrs. Next, 50 µl Bright-Glo luciferase reagent (Promega) was added, incubated for 5 min at room temperature and luminescence measured using a GloMax 96 luminometer and Relative Luminescence Units (RLU) per ml (auto-luminescence normalised using cell only control) determined.

**Pseudotype virus neutralization assay (PVNA)**

PVNAs were performed using a standard protocol (Temperton et al., 2007). Briefly, serially-diluted sera (1:40-1:200,000) were individually incubated with virus supernatant (2.5 × 10⁵ pfu/mL) and pseudotype virus particles.
RLU per well, calculated from the titration result) for 1 hr at 37°C to permit antibody attachment to virus particles. Next, $1 \times 10^4$ cells were added to each well, incubated for 48 hrs, and their luminescence read as described above. Test sample results were normalised by deducting any background luminescence produced by cell-only controls (no pseudotype virus). Additionally, the no-serum control (cells plus viruses) was included (equivalent to 0% neutralization). 

IC$_{50}$ antibody titres (the reciprocal of the serum dilution giving 50% inhibition of pseudotype virus entry) were calculated using GraphPad Prism computer software. Values <80 were considered negative (Katz et al, 1999; Garcia and Lai, 2011). Average values of two independent experiments are shown here.

**Single Radial Haemolysis (SRH) assay**

The SRH assay was performed as described in the OIE (World Organisation for Animal Health) Terrestrial Manual (OIE, 2012) using A/equine/Sussex/89 (H3N8) as antigen.

**RESULTS**

**Production of equine influenza pseudotyped virus using TMPRSS2**

In order to generate infectious H3 subtype equine influenza pseudotyped virus (EIPV) particles, it was necessary to co-transfect a plasmid expressing the TMPRSS2 endoprotease (transmembrane protease, serine S1 family member 2) to cleave the HA. No detectable virus was produced in the absence of this plasmid. The EIPV supernatant produced had a titre of $1 \times 10^9$ RLU/ml.

**Measurement of neutralizing antibodies in equine sera using pseudotyped virus**

The EIPV-containing supernatant produced was used to assay 20 equine serum samples (normalized data shown in Table 1). All samples from vaccinated animals exhibited IC$_{50}$ antibody titres of >1100 and the positive control serum showed strong neutralization, with an IC$_{50}$ of >40,000. The antibody titres of both serum samples from influenza-naïve animals were <80, the cut-off for a negative result.

**Measurement of virus-specific antibodies by single radial haemolysis**

Antibody was detected by SRH in the positive control serum and 18 of the 20 (90%) of the field sera. The SRH antibody levels ranged from 61-207 mm$^2$ (Table 1).

**Correlation of PVNA and SRH results**

Data from the two assays was compared using GraphPad Prism software. Pearson analysis (assuming a normal/Gaussian sample distribution) revealed a significant ($p = 0.002$) 65% correlation ($r$ value of 0.65) between the results.

**DISCUSSION**

Currently, the standard serological assays for animal and human influenza serology (e.g., assessment of vaccine efficacy and field epidemiology) are haemagglutination inhibition (HI), single radial haemolysis (SRH) and micro-neutralization (MN). Although well established, they are often labour-intensive, suffer from consistency problems and use native pathogenic viruses, sometimes requiring...
careful handling in containment facilities of biosafety level (BSL) 3 or above.

Retrovirus pseudotype technology has been used for diagnostic purposes and for basic research into a number of pathogenic RNA viruses that otherwise would have to be handled in BSL3/4 laboratories. These include Ebola virus (Wool-Lewis and Bates, 1998; Ito et al, 2001), hepatitis C (Bartosch et al, 2003; Hsu et al, 2003), SARS coronavirus (Nie et al, 2004; Tempertton et al, 2005), rabies and lyssaviruses (Wright et al, 2008; 2009; 2010) as well as avian influenza viruses (Tempterton et al, 2007). In contrast to the native viruses, their pseudotype counterparts can be handled in BSL1/2 facilities.

In this ‘proof-of-principle’ study, we firstly aimed to produce a high-titre EIPV as a research tool. Secondly, we tested whether this H3 subtype EIPV could be used to detect neutralizing antibodies in equines inoculated with a vaccine containing H3 subtype influenza viruses. Comparison was made to the established serological assay, SRH.

During natural infection, the newly synthesised influenza virus haemagglutinin protein (HA0) is cleaved by specific endoproteases into HA1 and HA2 subunits, to allow entry into susceptible cells (reviewed in Steinhauer, 1999; Garten and Klenk, 2008). The HA1 mediates binding to the sialic acid receptor, while HA2 is involved in membrane fusion. A number of proteases have been implicated in this cleavage process. The HA0 of highly-pathogenic avian influenza (HPAI) viruses of the H5 and H7 subtypes (e.g., A/Viet Nam/1203/2004 (H5N1)) have a polybasic cleavage site containing multiple arginine and lysine residues that are cleaved by ubiquitous subtilisin-like endoproteases such as furin (reviewed in Steinhauer, 1999). All other influenza A viruses, including low pathogenicity avian influenza viruses, seasonal human influenza and H3 equine influenza viruses, have monobasic cleavage sites with a single lysine or arginine residue (Figure 2). It has been shown that cleavage at these sites is mediated by specific type II transmembrane serine proteases such as TMPRSS2 and HAT (human airway trypsin-like protease) found in human airways (Böttcher et al, 2006; 2009). Consequently, we included a plasmid expressing the TMPRSS2 protease in the transfection procedure to mediate cleavage of the monobasic cleavage site in the equine pseudotype virus. By this approach, a high titre EIPV was successfully generated.

The pseudotype virus neutralization assay (PVNA) detected neutralizing antibodies in all of the sera from vaccinated equines and both negative controls had an IC₅₀ of <80. There was general agreement between the antibody levels measured by PVNA and SRH, although two serum samples in which no antibodies were detected by SRH displayed a moderate neutralizing antibody level as measured by PVNA.

Previous studies have shown good correlation between influenza-specific antibody titres in equine sera after natural infection with influenza measured by SRH and virus neutralisation assays (Morley et al, 1995). Furthermore, SRH antibody levels have been shown to correlate with protection after subsequent challenge (Mumford and Wood, 1992). Though antibodies detected by SRH are largely neutralizing (Morley et al, 1995), there are differences between SRH and neutralization assays such as PVNA and micro-neutralization (MN). For HA, SRH primarily detects responses resulting from antibody interactions with the head of the trimer (though it does detect other viral proteins), whereas both neutralization assays can also detect neutralizing antibodies in equines inoculated with a vaccine containing H3 subtype influenza viruses. Comparison was made to the established serological assay, SRH.

Table 1. Equine influenza H3N8 subtype-specific antibodies in 20 equine sera as measured by pseudotype virus neutralization assay (PVNA) and single radial haemolysis (SRH). Shading used to indicate relative antibody levels of response (SRH: ≤100 mm² = light grey, 100-150 mm² = medium grey, ≥150 mm² = dark grey; PVNA: ≤5000 = light grey, ≥1000 ≤10000 = medium grey, ≥10000 = dark grey).

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>PVNA (IC₅₀)</th>
<th>SRH (mm²)</th>
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<tbody>
<tr>
<td>1</td>
<td>1124</td>
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<tr>
<td>2</td>
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<td>Positive control</td>
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<tr>
<td>Negative control 1 ≤80</td>
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<tr>
<td>Negative control 2 ≤80</td>
<td>≤0.5</td>
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</tr>
</tbody>
</table>

Figure 2. Alignment showing the monobasic cleavage site of the equine influenza virus strain used in the study, A/1/equine Sussex/89 (H3N8), compared with the same region of representative avian and human influenza strains. Note the polybasic cleavage site in A/Viet Nam/1203/2004 (H5N1) strain not present in the others. The red box indicates the conserved cleavage site region, with the red-highlighted arginine (R) residue indicating the cleavage point. The green box shows the ‘fusion peptide’ allowing virus penetration of host cell endosomal membranes.

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efficiently detect responses to the HA stalk (Corti et al., 2011). This may explain the two PVNA positive but SRH negative results. In a comparison of SRH, neutralization using swine embryo kidney (ESK) cells and HI tests using serum samples from horses that had received two doses of vaccine, Yamagishi et al. (1982) found reasonable correlation between the assays. The correlation between the SRH assay and the neutralisation test was 75% ($r = 0.75$). This is comparable to the 65% correlation ($r = 0.65$) seen between SRH and PVNA in the present study. Yamagishi et al also found that the SRH assay was less sensitive than both the HI and neutralisation tests. Our data similarly indicates the slightly greater sensitivity of a neutralization assay using pseudotype viruses compared with SRH.

The PVNA will be further developed by assessment of the sensitivity and specificity of the assay. We also intend to test the robustness and reproducibility of the assay by evaluating inter- and intra-laboratory variability. Finally, a protective titre will be determined using sera obtained from vaccination and challenge studies.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Ann Cullinane (Irish Equine Centre, Johnstown, Ireland) for the negative control sera. This work was funded by the University of Kent and University of Nottingham.

COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

BSL; biosafety level
EIPV; equine influenza pseudotype virus
HA; haemagglutinin
HI; haemagglutination inhibition
MN; micro-neutralization
NA; neuraminidase
PV; pseudotype virus
PVNA; pseudotype virus neutralization assay
RLU; relative luminescence units
SRH; single radial haemolysis
TMPRSS2; transmembrane protease serine S1 family member 2
VN; virus neutralising

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