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 lentivirus 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 luciferase 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 haemolysis (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 common 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 variation 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 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 have been pseudotyped and used in such serological assays (Temperton et al, 2007; Temperton and Wright, 2009; Corti et al, 2011; Garcia and Lai, 2011). A pseudotype virus has the 'core' of one virus (*e.g.*, a retrovirus) and the outer 'envelope' protein of another (*e.g.*, the HA of influenza virus). The core virus has deletions in the genome making it replication-deficient, and harbours a reporter transgene (*e.g.*, luciferase). The envelope glycoprotein permits entry into susceptible target cells. During cell transduction, the pseudotype virus (PV) genome becomes integrated in the cell genome and expresses the reporter gene. Thus the number of transduced cells can be quantified and the subsequent inhibitory effects of antibodies in serum on PV entry determined (Temperton et al, 2007; Temperton and Wright, 2009; Garcia and Lai, 2011).

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 hyper-immunised 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 QIAmp 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'-GCGCGCGGATCCGTCAAAATGAAGACA ACCATTATT-3'

Reverse: 5'-GCGCGCCTCGAGAATTTAAATGCAAATG TTGCATCT-3'

PCR fragments were then digested with *Bgl*II and *Xho*I restriction enzymes and cloned into the pI.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 4×10^6 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% CO_2 (v/v) humidified incubator for 24 hrs. Next, the pI.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 (Böttcher 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 1×10^4 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 (2.5×10^5
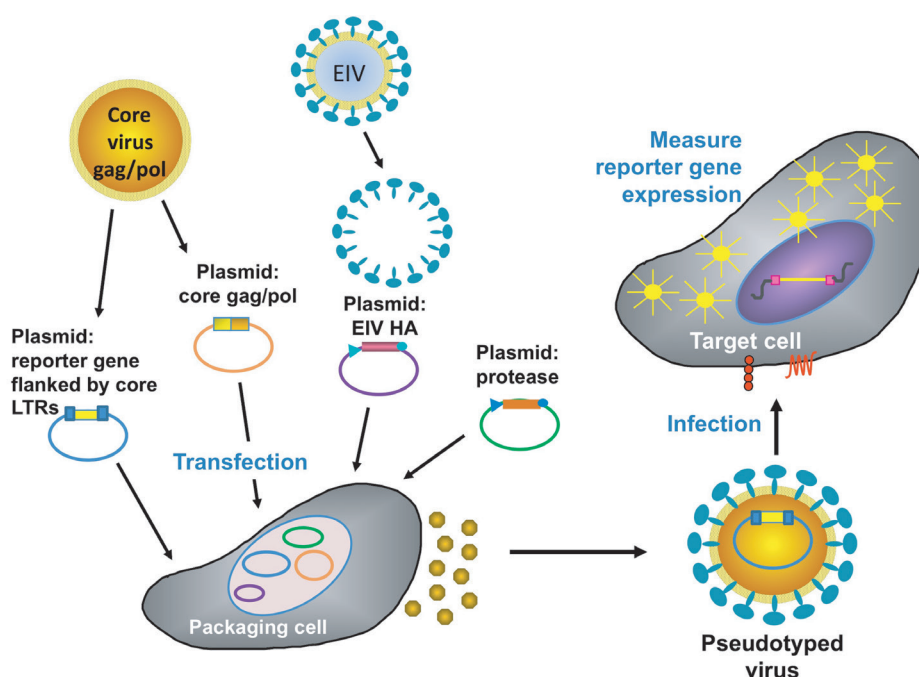
Figure 1. Schematic of method for generation and titration of equine influenza HA pseudotyped lentivirus. Four plasmids expressing the equine influenza virus (EIV) haemagglutinin (HA), HIV gag-pol, firefly luciferase reporter gene and TMPRSS2 protease are transfected into HEK293 cells. EIV pseudotyped lentivirus is harvested from virus supernatant, added to target 293 cells and relative transduction titre calculated from the level of reporter gene expression.

RLU per well, calculated from the titration result) for 1 hr at 37°C to permit antibody attachment to virus particles. Next, 1×10^4 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 1×10^9 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 mm² (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; Temperton et al, 2005), rabies and lyssaviruses (Wright et al, 2008; 2009; 2010) as well as avian influenza viruses (Temperton 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 IC_{50} 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

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, ≤10000 = medium grey, ≥10000 = dark grey).

Serum sample	PVNA (IC_{50})	SRH (mm²)
1	1124	79
2	1860	0
3	4521	0
4	4627	83
5	4673	112
6	6136	118
7	6308	116
8	7500	100
9	7637	140
10	7863	61
11	8056	118
12	9179	118
13	9837	106
14	10523	58
15	11768	147
16	13237	153
17	17908	170
18	22843	207
19	35422	156
20	46617	181
Positive control	40824	136
Negative control 1	≤80	≤0.5
Negative control 2	≤80	≤0.5

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A/equine/Sussex/1/1989(H3N8)   322 KYIRQNTLKLATGMRNV PEKQ----I R GIFGAIAGFIENGW EGMVDGWYGF 368
A/duck/Ukraine/1/63(H3N8)      323 KYVKQNTLKLATGMRNV PEKQ----T R GLFGAIAGFIENGW EGMIDGWYGF 369
A/HongKong/1/1968(H3N2)        323 KYVKQNTLKLATGMRNV PEKQ----T R GLFGAIAGFIENGW EGMIDGWYGF 369
A/Udorn/307/1972(H3N2)         323 KYVKQNTLKLATGMRNV PEKQ----T R GLFGAIAGFIENGW EGMIDGWYGF 369
A/VietNam/1203/2004(H5N1)      320 KYVKSNRLVLATGLRNS PQRERRRKK R GLFGAIAGFIEGGW QGMVDGWYGY 370
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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.

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

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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