The use of equine influenza pseudotyping for serological screening

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INTRODUCTION

The primary protective immune response against equine influenza virus infection is generation of neutralising antibodies against the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). As for human influenza, measurement of anti-HA antibodies is important in field studies and experimental vaccination and challenge studies to determine the effectiveness and efficacy , respectively, of vaccines.

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-lab variation are observed, as a result of which protective antibody levels are ill-defined, ranging from titres of 8 to 128 [1, 2]. Furthermore, the test only measures inhibition of HA binding to cells. The single radial haemolysis (SRH) assay measures complement-mediated haemolysis induced by influenza antibody–antigen complexes. SRH can be standardised using reference sera [2, 3] and a clear relationship has been demonstrated between pre-challenge antibody levels measured by SRH and protection afforded in challenge studies [1, 2]. 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 and awkward and the lack of cytopathic effect in cells 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 [2, 4–6]. A pseudotype virus has the 'core' of one virus (e.g. a retrovirus) and outer 'envelope' of another (e.g. the HA of influenza virus; Figure 1). The core virus has deletions in the genome making it replication-deficient, and harbours a transgene (e.g. luciferase). The envelope contains specific proteins from the study virus, which permit 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 and pseudotype entry determined [2, 4–6].

AIM

To develop a pseudotype virus neutralization assay (PVNA) for the measurement of neutralising antibodies to H3 subtype equine influenza viruses and compare with a standard serological assay (SRH)

MATERIALS AND METHODS

Viruses and sera

Stocks of equine influenza A/Sussex/89 virus were grown in 10-day-old embryonated hens' eggs. Serum samples surplus to diagnostic requirements were obtained from donkeys recently inoculated (>80 days prior) with vaccines 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 positive control was 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). Negative control horse sera were confirmed by SRH (kind gift of Dr Ann Cullinane, Irish Equine Centre)

Generation of H3 pseudotyped virus

Viral RNA was extracted from allantoic fluid using the QIAmp viral RNA extraction kit (Qiagen). The full HA gene was amplified by RT-PCR using custom primers (Invitrogen), and cloned into the pI.18 expression plasmid. Influenza pseudotyped lentivirus particles expressing a luciferase reporter gene were generated using four-plasmid transfection (Fugene6; Promega) into HEK293T cells [3]; pI.18-HA, p8.9-HIV-gag/pol, pCSFLW-Luc and a pCAGGS-TMPRSS2 protease plasmid to effect cleavage of the HA (Figure 1).

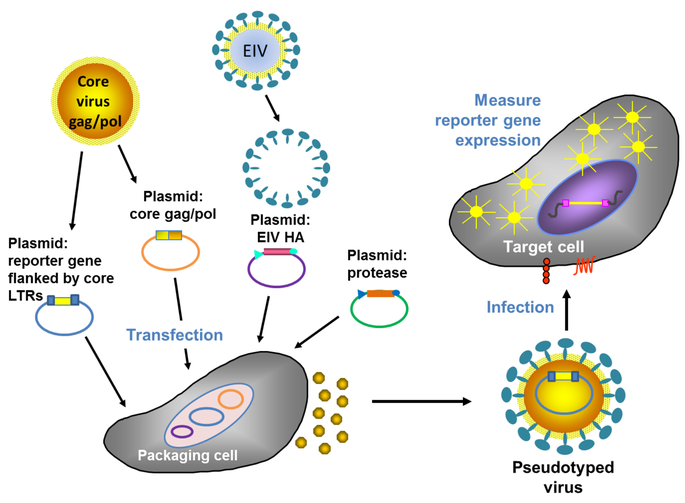

Figure 1: Schematic of four-plasmid system for generating equine influenza HA pseudotyped virus

Serological assays

Pseudotype virus neutralization assays (PVNAs) were performed using a standard protocol with HEK293T target cells and Bright-Glo (Promega) for luciferase measurement [4]. Serum samples were tested in duplicate.

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

RESULTS

An equine influenza pseudotype virus was successfully generated and used to assay 20 post-vaccination equine serum samples (Table 1).

Serum sample	PVNA (IC_{50})	SRH (mm^2)
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

Table 1: Equine influenza H3 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 ≤100mm2 light, 100-150mm2 medium, ≥150mm2 dark; PVNA ≤5000 light, ≤10000 medium, ≥10000 dark. NB: PVNA titres <80 are considered negative.

CONCLUSIONS & FUTURE WORK

- TMPRSS2 protease necessary for HA cleavage in PV production
- The PVNA shows promise for the measurement of neutralising antibody responses. Assay correlation was good (r=65%, p=0.0002; Pearson analysis using GraphPad)
- PVNA and SRH measure different types of antibody responses with differing sensitivity
- Further development of the assay will include:
  - Assessment of sensitivity/specificity and inter/intra-laboratory variability
  - Definition of a protective titre (sera obtained from vaccination and challenge studies)
- For further details of current study see [7] below

REFERENCES

[1] Mumford & Wood (1992) *Develop. Biol. Stand.* 79:137–46

[2] Montomoli *et al.* (2011) *Influenza vaccines for the future* (2nd Ed.) 199–209

[3] Daly *et al.* (2007) *Pharmeuropa Bio* 1:7–14

[4] Temperton *et al.* (2007) *Influenza and Other Respiratory Viruses* 1:105–12

[5] Temperton & Wright (2009) *Encyclopedia of Life Sciences* 10

[6] Garcia & Lai (2011) *Expert Rev. Anti Infect. Ther.* 9:443–455

[7] Scott *et al.* (2012). *J. Genet. Mol. Med*. 6:304-308

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