

Influenza A viruses with truncated NS1 as modified live virus vaccines: Pilot studies of safety and efficacy in horses

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Supplementary materials and methods

Experimental animals

All animals were raised from birth at the research farm of the Department of Veterinary Science, University of Kentucky, and their influenza status was known. *Trial I* (safety trial) included both influenza-experienced (seropositive) and influenza-naïve horses/ponies of 8 months–2 years age. *Trial II* (effectiveness trial) utilised influenza-naïve yearling horses.

Clinical monitoring

For initial evaluations of safety (*Trial I*) and also for assessment of clinical protection following experimental challenge with wild-type virus (*Trial II*), physical examinations were performed on all horses daily from one (Day -1) or more days before to 8 days after virus administration (Day +8). Examinations included measurements of rectal temperature (fever was defined as >38.5°C), respiratory rate, stethoscopic audition of lung sounds, palpation of submandibular and parotid lymph nodes, general

demeanour and presentation of nasal discharge or spontaneous coughing. A clinical scoring system was used, which was similar to that previously described (Chambers *et al.* 2001), except that lung sounds were also incorporated: 5 sectors on each side of the animal were evaluated on a 0 to 5 scale, and the mean of the 10 sector scores was added to the other clinical scores (Table 1). Examinations and clinical scoring were done by a veterinarian (T.S.) blinded to the vaccination status of the horses. For the vaccination portion of *Trial II*, clinical observations consisted of rectal temperature and visible signs only on Days 0 to +4.

Serological analysis

In *Trial I*, sera were collected on the day of vaccination and weekly thereafter for 4 weeks. In *Trial II*, sera were collected on both days of vaccination (V1 and V2, 4 weeks apart), and again collected on the day of challenge and weekly thereafter for 3 weeks. Sera were tested for antibodies to the H7N7 prototype equine influenza virus Prague/56 and the H3N8 strains Newmarket/2/93 (European lineage), Kildare/92 (American lineage) and South Africa/03 (American lineage) by single radial haemolysis (SRH) (Anon 2004). A positive control serum purchased from the European Directorate for the Quality of Medicines and Healthcare (EDQM) was included on each immunodiffusion plate. All sera were tested for nonspecific lysis. SRH antibody levels were expressed as mm² of haemolysis. Seroconversion was defined as an increase in SRH value of >25 mm² or 50%, whichever was the smaller value (Newton *et al.* 2000).

Viral shedding and confirmation of genotype

Nasopharyngeal secretion samples were collected via swabs inserted 15–25 cm into the nasal meatus. Swabs were taken daily on days of vaccination and for 8 days (*Trial I*) or 3 days (*Trial II*, V1 and V2) following, and on day of challenge and for 8 days following. Viral shedding was detected by virus isolation in embryonated hen eggs (Anon 2004). Secretions collected post vaccination were subject to RT-PCR so the genotype of any virus being shed could be confirmed. Viral RNA was isolated from 140 µl of nasal secretions using the QIAamp Viral RNA Mini kit¹ according to the manufacturer's recommendations. RT-PCR was carried out with primers that amplified the entire NS gene so that the 3 recombinant NS1 viral genotypes could be distinguished

TABLE 1: Clinical signs scoring index

Clinical sign	Degree	Score
Coughing	No coughing	0
	Coughed once	1
	Coughed twice or more	2
Nasal discharge	No discharge	0
	Abnormal serous	1
	Abnormal mucopurulent	2
Respiration	Abnormal profuse	3
	Normal <36/min	0
	Abnormal (dyspnoea/tachypnoea) >36/min	1
Demeanour	No depression	0
	Depression present (lethargy, inappetence)	1
Lymph nodes	Normal	0
	Enlarged	1
	Draining	2
Lung score	Σ quadrant scores/10, rounded to nearest whole number	
	Normal	0
	Slight inspiratory wheeze	1
	Strong inspiratory wheeze	2
	Inspiratory/expiratory wheeze	3
	Crackles	4
Pleuropneumonia	5	

(Quinlivan *et al.* 2005a). One step RT-PCR was performed using the Access RT-PCR system kit² according to the manufacturer's recommendations. Primers were used at a final concentration of 0.5 µmol/l. Thermocycler conditions consisted of reverse transcription at 45°C for 40 min followed by denaturation at 95°C for 3 min. cDNA was amplified with 35 cycles of 95°C for 30 s, 55°C for 1 min and 72°C for 1 min with a final extension at 72°C for 5 min. RT-PCR products were separated on a 1.2% agarose gel by electrophoresis to confirm the genotype of the virus being shed.

Viral shedding quantitation

Virus shedding post challenge was quantified by 50% egg infectious dose (EID₅₀) analysis (Karber 1931; Anon 2004), and by quantitative RT-PCR (qRT-PCR). One step RT-PCR was performed using Light Cycler RNA Amplification kit, SYBR Green I³ as previously described (Quinlivan *et al.* 2005b) with some modifications. The same reaction conditions were used apart from an increase in temperature for acquisition of fluorescence data from 72 to 83°C to eliminate nonspecific fluorescence measurements. The same reaction mix and primers M52C and M253R (Fouchier *et al.* 2000) were employed. Viral genomic RNA from the challenge virus strain (A/eq/KY/2002) was used as

a standard. Its concentration was determined by OD₂₆₀ and the genome copy number was calculated. Serial log dilutions of the RNA were used to generate a standard curve ranging from 3 x 10⁶ to 300 copies of RNA. The amount of RNA in the unknown samples was then determined by interpolation and corrected to RNA copies per ml of nasal secretions.

Inflammatory cytokine response post challenge

Venous blood samples were taken on the day prior to challenge and for 6 days post challenge. These were collected into PAXgene¹ tubes which contain a stabilising additive to preserve the RNA expression profile (Rainen *et al.* 2002). RNA was isolated from PAXgene bloods as per manufacturer's recommendations and quantified by OD₂₆₀. Quantitative RT-PCR was carried out on the ABI TaqMan 7500 platform⁴ and data were analysed as a relative quantification study (Livak and Schmittgen 2001) using the system software. The sample taken prior to challenge (Day -1) was chosen as the calibrator sample. Relative quantification was then used to compare gene expression levels post challenge to the day prior to challenge. Equine β-glucuronidase (β-GUS) was used as an endogenous control to normalise for differences in RNA and cDNA. This is a putative housekeeping gene, is present at a consistent expression level in experimental samples and does not have processed pseudogenes (Shipley *et al.* 1991; Aerts *et al.* 2004; Dheda *et al.* 2004). FAM-labelled primer probes for IFNγ, IL-1β, IL-6, TNF-α and β-GUS were designed using the Assays-By-Design service⁴ (Table 2). The 2^{-ΔΔCT} method for analysing relative gene expression from real-time quantitative PCR experiments was first validated for each of the primer probes sets and employed for analysis of data (Livak and Schmittgen 2001). Reverse transcription of 0.5 µg RNA was carried out using 1 µmol/l of oligo dT primer⁵, 0.3 units of AMV reverse transcriptase³, and 0.5 units of RNAsin², in a thermocycler⁶ for 50 min at 42°C followed by 95°C for 5 min. TaqMan PCR conditions employed were 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was tested in triplicate for each of the cytokine targets and the endogenous control.

Statistical analysis

Microsoft SPSS 13.0 was used for all statistical tests carried out. ANOVA was used to analyse rectal temperatures post challenge,

TABLE 2: Primer probe sequences for cytokine gene expression analysis

Name	Sequence (5' to 3')
EqIL-1B Fwd	CCGACACCAGTGACATGATGA
EqIL-1B Rev	ATCCTCCTCAAAGAACAGGTCATTC
EqIL-1B Probe	ATTGCCGCTGCAGTAAG
EqIL-6 Fwd	GGATGCTTCCAATCTGGGTTCAAT
EqIL-6 Rev	TCCGAAAGACCAGTGGTGATTTT
EqIL-6 Probe	ATCAGGCAGGTCTCCTG
EqTNFα Fwd	TTACCGAATGCCTTCCAGTCAAT
EqTNFα Rev	GGGCTACAGGCTTGTCACCTT
EqTNFα Probe	CCAGACACTCAGATCAT
EqIFNγ Fwd	AGCAGCACCAGCAAGCT
EqIFNγ Rev	TTTGCCTGGACCTTCAGA
EqIFNγ Probe	ATTCAGATTCGGTAAATGA
EqGUS Fwd	GCTCATCTGGAACCTTGTGATTTT
EqGUS Rev	CTGACGAGTGAAGATCCCTTTT
EqGUS Probe	CTCTCTGCGGTGACTGG

TABLE 3: Trial II SRH antibodies post vaccination/challenge

V	V1				V2				C0				C7				C14				C21			
	N	K	SA	P	N	K	SA	P	N	K	SA	P	N	K	SA	P	N	K	SA	P	N	K	SA	P
1	0	0	0	0	108	124	120	0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	0	0	0	0	102	115	104	0	53	80	81	0	75	82	90	0	148	177	168	0	125	146	139	0
3	0	0	0	0	103	109	93	0	67	73	70	0	79	103	99	0	160	209	180	0	159	194	167	0
4	0	0	0	0	108	135	107	0	98	130	105	0	87	117	112	0	105	125	121	0	87	111	110	0
5	0	0	0	0	116	124	108	0	74	95	101	0	86	112	102	0	172	243	207	0	187	221	206	0
6	0	0	0	0	65	102	80	0	47	53	58	0	163	190	164	0	241	354	259	0	221	290	255	0
C																								
1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	227	309	262	0	208	268	222	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	218	264	193	0	183	240	178	0
3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	210	296	233	0	175	234	175	0

V1, V2: blood was collected prior to administration of V1 and V2 vaccinations. C0–C21, blood was collected on the indicated day post challenge, with C0 being the day of challenge. N = Newmarket/2/93; K = Kildare/92; SA = South Africa/03 and P = Prague/56. ND, this horse was not included in the challenge.

cytokine gene expression and antibody titres post challenge. The Mann Whitney U test was used for analysis of clinical data post challenge. The unpaired *t* test was used to analyse differences between vaccinated and control groups. P values <0.05 were deemed to be statistically significant.

Manufacturers' addresses

¹Qiagen, Valencia, California, USA.

²Promega Corporation, Madison, Wisconsin, USA.

³Roche, Basel, Switzerland.

⁴Applied Biosystems, Foster City, California, USA.

⁵Invitrogen, Carlsbad, California, USA.

⁶Perkin Elmer Cetus, Emeryville, California, USA.

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