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# A recombinant Hendra virus G glycoprotein-based subunit vaccine protects ferrets from lethal Hendra virus challenge

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ABSTRACT

The henipaviruses, Hendra virus (HeV) and Nipah virus (NiV), are two deadly zoonotic viruses for which no vaccines or therapeutics have yet been approved for human or livestock use. In 14 outbreaks since 1994 HeV has been responsible for multiple fatalities in horses and humans, with all known human infections resulting from close contact with infected horses. A vaccine that prevents virus shedding in infected horses could interrupt the chain of transmission to humans and therefore prevent HeV disease in both. Here we characterise HeV infection in a ferret model and show that it closely mirrors the disease seen in humans and horses with induction of systemic vasculitis, including involvement of the pulmonary and central nervous systems. This model of HeV infection in the ferret was used to assess the immunogenicity and protective efficacy of a subunit vaccine based on a recombinant soluble version of the HeV attachment glycoprotein G (HeVsG), adjuvanted with CpG. We report that ferrets vaccinated with a 100 μg, 20 μg or 4 μg dose of HeVsG remained free of clinical signs of HeV infection following a challenge with 5000 $TCID_{50}$ of HeV. In addition, and of considerable importance, no evidence of virus or viral genome was detected in any tissues or body fluids in any ferret in the 100 and 20 μg groups, while genome was detected in the nasal washes only of one animal in the 4 μg group. Together, our findings indicate that 100 μg or 20 μg doses of HeVsG vaccine can completely prevent a productive HeV infection in the ferret, suggesting that vaccination to prevent the infection and shedding of HeV is possible.



## 1. Introduction

Hendra virus (HeV) is a zoonotic virus transmitted from bats to humans via horses. While HeV related disease in bats has not been documented, the virus can cause a severe systemic illness, with severe pathology associated with the respiratory and neurological systems in both horses and humans [1]. Four of the seven human infections recorded so far have been fatal and the disease is usually fatal in horses – in the first recorded outbreak of HeV in 1994 in Brisbane, Queensland, Australia 14 horses died out of a total of 20 infected with HeV [2]. Including the initial outbreak there have been 14 known spillovers of HeV and all except one (in northern New South Wales) occurred in Queensland [3].

HeV is one of only two members of the genus *Henipavirus* in the family *Paramyxoviridae* [4,5]. The henipaviruses are characterised by a large genome and their ability to infect a wide range of animals, including humans. The other member of the genus, Nipah virus (NiV), was first isolated from a disease outbreak that occurred in Malaysia in 1998 in humans and pigs [6]. Out of 265 human cases, 105 were fatal. Since 2001 there have been numerous NiV outbreaks in Bangladesh and two in India [7], the most recent occurrence in early 2011, in Bangladesh [8]. At least two outbreaks have been associated with virus transmission from human-to-human [9–11] with both respiratory and neurological signs observed in humans, and mortality rates ranging from 40% to 75%.

As a result of the potential for henipaviruses to cause significant mortality and morbidity in humans they are classified as Biosafety Level 4 (BSL-4) agents. Further, due to their carriage by wildlife and their relative ease of propagation, the henipaviruses are considered select agents of concern for biodefense by the Centers for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID). In spite of this no licensed prophylactic or therapeutic treatments are currently available although several therapeutic modalities are under active investigation.

Like most paramyxoviruses, henipavirus infection of host cells involves two viral glycoproteins [12]. The G glycoprotein is the viral

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attachment protein and exists as a tetramer embedded in the lipid membrane of the virus. Henipavirus G binds to the host cell receptors; ephrin-B2 and ephrin-B3 [13–16], important bi-directional cell–cell signalling molecules that are highly conserved and widely expressed particularly within the nervous and vascular systems [17] across all mammalian species. The second viral glycoprotein is the fusion (F) protein, which upon triggering facilitates the fusion between the viral and cellular membranes.

An immune response to viral surface proteins/glycoproteins is often necessary for resistance to viral infection [18] and is particularly effective in controlling infections with a viraemic phase such as the human paramyxoviruses that cause mumps and measles [19,20]. Similarly, passive protection against NiV infection has recently been demonstrated in a ferret model by transferring a G glycoprotein specific, HeV and NiV cross-reactive, human monoclonal antibody [21] and in a hamster model by transferring G or F glycoprotein specific polyclonal or monoclonal antibody [22–24]. In the hamster model, vaccination with recombinant vaccinia viruses expressing G or F also induced protection against a lethal challenge with NiV [22]. A similar outcome has been demonstrated in pigs vaccinated with a canarypox vaccine carrying G or F [25]. In two different experiments cats vaccinated with a soluble G glycoprotein (sG) based subunit immunogen survived a lethal NiV challenge with no clinical signs [26,27]. Although no clinical disease was observed, in one experiment genome was detected in oral swabs, urine and the brain of several animals, virus was isolated from the brain of one animal [26] and in the other experiment genome was detected in the tissues of two animals at levels that were so low as to be questionable [27]. There is 83.3% identity between the amino acid sequences of the HeV and NiV G glycoproteins [28] and it has been shown that immunisation with sG of either HeV or NiV produces cross neutralising antibodies, with a better cross neutralisation response elicited by HeV soluble G (HeVsG) [27]. HeVsG therefore has potential as a subunit vaccine immunogen for preventing both HeV and NiV infection.

Previous studies have revealed that ferrets are a very successful model for NiV infection, closely mirroring the characteristics of the infection in humans [21,29]. NiV infected ferrets exhibit severe respiratory and neurological disease as well as generalised vasculitis. Here, we have evaluated HeV infection of ferrets and extend the use of this new animal model to assess the protective efficacy of HeVsG as a vaccine immunogen against lethal HeV challenge. We show that, like NiV, the manifestation of HeV infection and pathogenesis in ferrets is similar to that seen in humans exhibiting both respiratory and neurological disease. Further, in this model system, the three HeVsG vaccine doses tested prevented clinical disease after a lethal HeV challenge, and following the two higher doses of immunogen there was no detectable evidence of HeV infection.

## 2. Materials and methods

### 2.1. Animals, accommodation, handling, and biosafety

Eight male ferrets aged 12–18 months were used for the HeV model development study and eight were used for the HeVsG vaccination study. The animal husbandry methods and experimental design were endorsed by the CSIRO Australian Animal Health Laboratory's Animal Ethics Committee. Animals were housed in a single room at BSL-4 in pairs in cages that incorporated two "squeeze" compartments for administration of chemical restraint, given a complete premium dry food and provided with water *ad libitum*. Room temperature was maintained at 22 °C with 15 air changes per hour; and humidity varied between 40% and 60%. Before any manipulation, animals were immobilized with a mixture of ketamine HCl (3 mg/kg; Ketamil; Ilium, Smithfield, Australia) and medetomidine (30 μg/kg; Domitor; Novartis, Pendle Hill, Australia) by intramuscular injection. For reversal, atimepazole (Antisedan; Novartis) was given intramuscularly at 50% of the dose used for medetomidine. At least one week prior to virus challenge single stage transmitters fitted with an internal loop antenna and coated with an inert two-pot epoxy resin (Sirtrack, Havelock North, New Zealand) were implanted subcutaneously in the flank of the ferrets for the purpose of real-time continuous monitoring of body temperature. Staff wore fully encapsulated suits with breathing apparatus while in the animal room. Serology, virus isolations, and the initial stages of RNA extraction were carried out at BSL-4.

### 2.2. Animal infections

Ferrets were exposed to a low passage isolate of HeV (Redlands 2008) by the oronasal route. For the HeV infection study, 2 ferrets per group were exposed to 50 $TCID_{50}$ (ferrets 1-50, 2-50), 500 $TCID_{50}$ (3-500, 4-500), 5000 $TCID_{50}$ (5-5000, 6-5000) or 50,000 $TCID_{50}$ (7-50,000, 8-50,000) and for the sG vaccination experiment ferrets were exposed to 5000 $TCID_{50}$ at day 41 of the experiment i.e. 21 days post the booster vaccination.

General clinical observations were documented daily prior to as well as after challenge. Animals were weighed while under sedation at the time of vaccination and challenge and at days 6, 8, 10, and 21 post-challenge (pc). Rectal temperature was also determined at sedation by using digital thermometers to augment data derived remotely from the implanted temperature transponders. Ferrets were euthanized when reaching a previously determined endpoint or 21 days pc. The humane endpoint was defined as rapidly progressive clinical illness of up to 2 days duration including fever and depression, possibly accompanied by increased respiratory rate or posterior paresis or ataxia. In susceptible animals, this typically occurs within the first 10 days after viral challenge. In preliminary studies, these signs were found to correlate with the requirement to euthanize ferrets on subsequent days on humane grounds; thus, they have been utilized as surrogates for lethality.

### 2.3. Vaccine immunogen preparation

A human codon optimized HeV soluble glycoprotein G (sG) construct was used to produce recombinant HeVsG. The construct was generated by cloning the entire ectodomain coding regions of HeV G linked to an IgK leader sequence and S-peptide tag into pcDNA CMV+hygro. The expression plasmid pcDNA-CMV+hygro was generated by insertion of the CMV promoter element from plasmid phCMV-1(Gelantis, San Diego, CA) into pCDNA3.1(hygro) (Invitrogen, Carlsbad, CA). A stable HeVsG secreting cell line was generated by transfecting plasmid pcDNA-CMV+hygro-HeVsG into human 293F cells and selection using hygromycin B followed by limiting dilution cloning, generating the cell line HeVsG#4-2 293F. HeVsG was prepared by growing HeVsG#4-2 293F cells in shaker cultures using serum-free medium- 293 SFM II (Invitrogen) and purified by S-protein agarose affinity chromatography followed by preparative gel filtration chromatography with a Hiload 16/60, Superdex 200 column. CpG oligodeoxynucleotide (ODN) 2007 (TCGTCGTTGTCGTTTTGTCGTT) containing a fully phosphorothioate backbone was purchased from Invivogen (San Diego, CA, USA) and Alhydrogel™ was purchased from Accurate Chemical & Scientific Corporation (Westbury, NY, USA). Although the CpG component of the adjuvant is species specific, in the absence of any information on ferret specific CpG sequences the same CpG component was used for the ferret vaccine as was used for cats [26]. Vaccine doses containing fixed amount of CpG ODN 2007 and varying amounts of HeVsG and aluminium ion (at a weight ratio of 1:25) were formulated as follows: 100 μg dose: 100 μg HeVsG, 2.5 mg aluminium ion and 150 μg of CpG ODN 2007; 20 μg dose: 20 μg

HeVsG, 500 μg aluminium ion and 150 μg of CpG ODN2007; and 4 μg dose: 4 μg HeVsG, 100 μg aluminium ion and 150 μg of CpG ODN 2007. For all doses, Alhydrogel™ and HeVsG were mixed first before CpG ODN 2007 was added. Each vaccine dose was adjusted to 1 ml with PBS and mixtures were incubated on a rotating wheel at room temperature for at least 2–3 h prior to injection.

### 2.4. Immunisation

Ferrets were divided into 4 groups of 2, with each group receiving either a 100 μg HeVsG (ferrets 1-100, 2-100), 20 μg HeVsG (ferrets 3-20, 4-20) or 4 μg HeVsG (ferrets 5-4, 6-4) dose of vaccine or adjuvant alone (ferrets 7-0, 8-0). The vaccine was administered subcutaneously with a priming dose at day 0 and a booster dose 20 days later. Blood samples were collected for antibody analysis at day 0, day 20 and day 30. Nasal washes, oral and rectal swabs, and blood samples in EDTA were taken before the first vaccination.

### 2.5. Sample collection

Nasal washes, oral and rectal swabs, and blood samples both in EDTA and for serum preparation, were taken before challenge and at days 6, 8, 10 and 21 pc. For the vaccination experiment blood was taken before challenge as detailed in Section 2.4. Urine was only collected on day 21 pc due to the tendency of ferrets to urinate on being woken. Rectal and oral swabs were collected in duplicate into 1 ml of PBS for virus isolation or 800 μl of MagMAX Lysis/Binding solution (Ambion, Victoria, Australia) for genome extraction. For urine, nasal washes and EDTA blood, 100 μl of fluid was added to 260 μl Lysis/Binding solution.

At post-mortem (PM) examination, tissues were collected for virus isolation, viral genome detection, histology and immunohistology. These tissues included adrenal gland, bladder, occipital lobe of the brain, olfactory pole, heart, kidney, liver, apical lung lobe, diaphragmatic lung lobe, bronchial lymph node, retropharyngeal lymph node, spleen and testes. Tissue samples were either collected into 1 ml of PBS containing 1 mm stainless steel beads (Biospec Products Inc., Bartlesville, OK, USA) for virus isolation, 800 μl MagMAX Lysis/Binding solution containing 1 mm stainless steel beads for genome extraction, or fixed in 10% neutral buffered formalin for 48 h prior to routine processing for histology. Immunohistochemical evaluation was carried out using a rabbit polyclonal antibody raised against the NiV N protein [30].

### 2.6. RNA detection and HeV TaqMan PCR assay

Tissue samples in MagMAX Lysis/Binding buffer were homogenised for 30 s in a Mini-Beadbeater (BioSpec Products Inc.) and centrifuged to pellet debris. 260 μl of homogenised sample was then extracted using the MagMAX-96 viral RNA isolation kit (Ambion). TaqMan real-time PCR was carried out using the AgPath-ID one-step reverse transcription-PCR kit (Applied Biosystems, Victoria, Australia), targeting the N gene of HeV using primers FOR (5′-GATATITTTGAMGAGGCGGCTAGTT-3′), REV (5′-CCCATCTCAGTTCTGGGCTATTAG-3′), and probe (6FAM-CTACTTTGACTACTAAGATAAGA-MGB). Positive results were defined by a cycle threshold (*CT*) value of <40 [31]. Relative quantification of viral RNA levels in the tissues of each animal relative to the occipital lobe of the brain was performed using the comparative $C_T$ or $\Delta\Delta C_T$ method [32]. The occipital lobe of the brain was chosen as the reference tissue because the relatively low viral RNA load meant that most tissues carried relatively more viral RNA, making a graph of the results easier to interpret.

### 2.7. Virus isolation

For virus isolation, supernatants from homogenized tissues positive for HeV genome were incubated on Vero cell monolayers and scored positive if syncytia, as a measure of viral cytopathic effect, were present after 6 days.

### 2.8. Serum neutralisation test

Serial doubling dilutions of sera were carried out (final volume 50 μl/well) to which 50 μl HeV (100 $TCID_{50}$) was added and incubated for 1 h at 37 °C. Following incubation 100 μl Vero cells at $2 \times 10^5$-cells/ml was added to each well and the assay was read after 3 days incubation in a humidified 5% $CO_2$ incubator.

### 2.9. Measurement of antibody to HeV F glycoprotein in ferret serum

Recombinant expressed HeV sF (Chan and Broder, in preparation for publication) was coupled to microspheres and multiplexed microsphere assays were performed essentially as previously described [33]. A Luminex*H* 100 ISTM machine and MiraiBio software (MiraiBio Group, South San Francisco, CA) were utilized for all assays: Bio-Plex Manager software (Bio-Rad Industries, Hercules, CA) for acquisition and analysis. All samples were assayed simultaneously and concentrations were extrapolated from a standard curve using non-linear regression analysis (GraphPadSoftware, San Diego, CA).

## 3. Results

### 3.1. HeV infection in ferrets

Ferrets have been shown to be acutely sensitive to NiV infection and pathogenesis [21,29]. The purpose of developing the HeV ferret model here was to determine the predicted susceptibility of these animals to HeV challenge and HeV-mediated pathogenesis and to derive a challenge dose of HeV that would reliably infect susceptible animals, induce disease that would be expected to be lethal without other intervention, and could be applied to subsequent vaccine and therapeutic studies. Ferrets in the different dose groups 1-50, 2-50, 3-500, 4-500, 5-5,000, 6-5,000, 7-50,000 and 8-50,000 were all infected and all ferrets reached a humane endpoint and were euthanized from days 6 to 9 pc. There was no association of virus dose with incubation time, clinical signs, time to endpoint, and either distribution or severity of gross or histopathological lesions. Fever was established in all animals by day 6 pc, and clinical signs included depression, lack of grooming, and generalised tremors. At post mortem examination, there was subcutaneous edema of the head and neck, cutaneous petechiation, numerous 1 mm slightly hemorrhagic nodules scattered throughout the parenchyma of the lung, together with marked hemorrhage of submandibular, bronchial, duodenal and mesenteric lymph nodes. On histologic examination ferrets showed systemic vasculitis, necrotising lymphadenitis, glomerulitis, splenitis and bronchoalveolitis with syncytial cell formation. HeV antigen was identified in meningeal endothelial cells (Fig. 1A and B), lymphatic, glomerular, splenic, pulmonary, cardiac, testicular, pancreatic and intestinal endothelial cells as well as bronchoalveolar epithelium (Fig. 1C).

### 3.2. HeV genome detection and virus isolation from infected ferrets

Thirteen different tissues from each of the 8 ferrets were screened for the presence of HeV RNA as were oral and rectal swabs, urine and blood. RNA was detected in all tissues tested from each

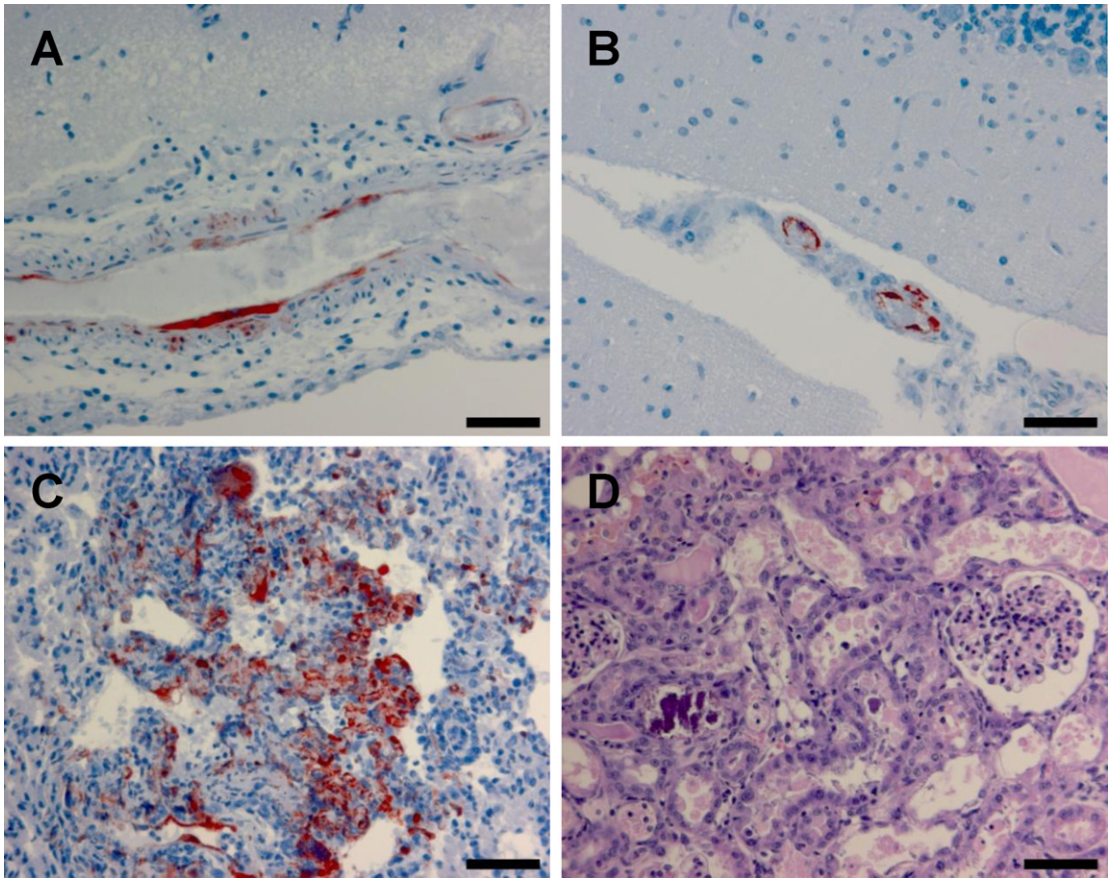


**Fig. 1.** Immunohistology using rabbit polyclonal anti-Nipah N protein. (A and B) demonstrating antigen in endothelium of meningeal blood vessels of ferrets 7 and 9 days post exposure to 5000 $TCID_{50}$ HeV (ferrets 5-5000 and 6-5000) and also (C) associated with acute necrotising alveolitis in ferret 6-5000 at 9 days post infection. (D) Post-challenge acute renal tubular necrosis not attributed to HeV in ferret 5-4 immunised with 4 μg HeVsG vaccine (Hematoxylin & eosin). Scale bar = 100 μm.

ferret. On average, for the 8 animals the lowest relative $C_t$ level and highest proportion of virus isolations was in the kidney, lung and spleen. Positive virus isolations were made in these tissues from all 8 ferrets. The highest relative $C_t$ level and the lowest level of virus isolation were observed in the occipital lobe of the brain, olfactory pole and the heart (Fig. 2). Virus was isolated from the occipital lobe at PM (day 9 pc) in ferret 1-50; from the olfactory pole at day 6 pc in ferret 4-500, at PM (day 9 pc) in ferret 1-50 and at PM (day 7 pc) in ferret 8-50,000; from the heart at PM (day 9 pc) in ferret 1-50.

For each sampling day genome was detected in all oral and rectal swabs, blood samples and in the 7 urine samples collected at post mortem (there was no urine sample from 2-50 at PM). However, the only virus isolations were from 3 urine samples at PM (day 7 pc) from ferrets 5-5000, 7-50,000 and 8-50,000, one blood sample at day 6 pc from ferret 2-50 and one rectal swab at PM (day 8 pc) from ferret 3-500.

### 3.3. Immunisation

No reactions such as swelling or erythema were identified at vaccine sites. At the time of the booster vaccination at day 20, neutralising titres in sera correlated to the dose of sG administered; ranging from a titre of 8192 for the 100 μg vaccination group, to 1024/2048 for the 20 μg vaccination group, and 64/128 for the 4 μg vaccination group (Table 1). A greater rise in SNT titre was noted at day 30 following the booster immunizations in the lower antigen groups, and the lack of a measurable rise in SNT titre in the 100 μg vaccination group was likely due to the larger amount of antigen used in the first immunization and the timing of the booster immunization. Prior to challenge, neutralising titres in all 6 vaccinated animals were 8192 or greater.

### 3.4. Hendra virus challenge of immunized ferrets

Ferrets in the vaccination and challenge study were inoculated with a low passage isolate of HeV (Redlands 2008) by oronasal administration. Based on the outcome of the minimal infectious dose experiment we chose a 5000 $TCID_{50}$ dose (100 times the minimal lethal dose observed) administered at 21 days post booster vaccination. Control ferrets vaccinated with adjuvant only and subsequently challenged with HeV (ferrets 7-0 and 8-0) became febrile (>40 °C) on day 5 pc, showed reduced play activity and hind limb paresis, and were euthanized on day 7 pc. All HeVsG immunised ferrets including the 100 μg dose group (ferrets 1-100, 2-100), the 20 μg dose group (ferrets 3-20, 4-20) and one of the 4 μg dose group

**Table 1**
HeV serum neutralisation titres from HeVsG vaccinated and control ferrets prior to challenge.

| Ferret # – dose HeVsG | HeV SNT titre | | |
|---|---|---|---|
| | Day 0 | Day 20 | Day 30 |
| 1-100 | <1:2 | 1:8192 | 1:8192 |
| 2-100 | <1:2 | 1:8192 | 1:8192 |
| 3-20 | <1:2 | 1:1024 | 1:8192 |
| 4-20 | <1:2 | 1:2048 | 1:16384 |
| 5-4 | <1:2 | 1:64 | 1:8192 |
| 6-4 | <1:2 | 1:128 | 1:8192 |
| 7-0 | <1:2 | <1:2 | <1:2 |
| 8-0 | <1:2 | <1:2 | <1:2 |

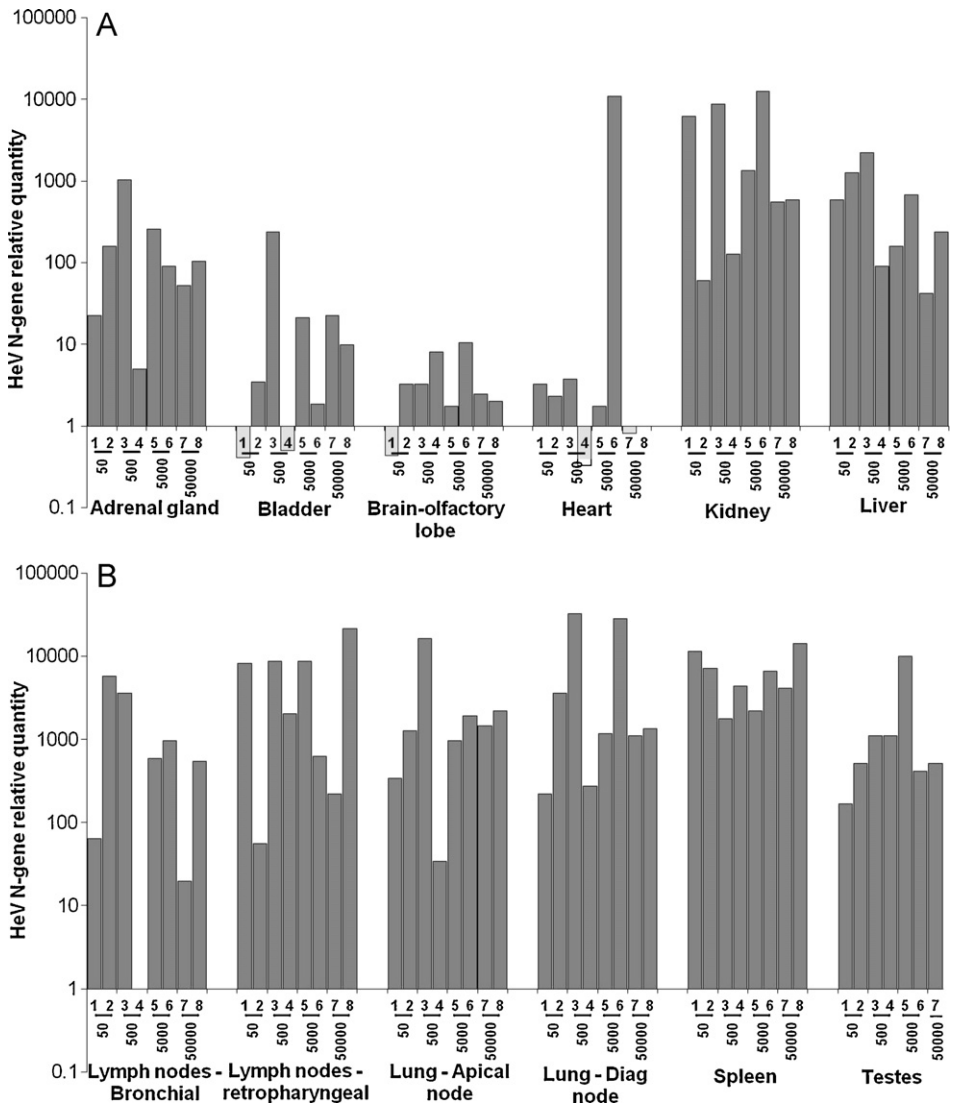


**Fig. 2.** Relative abundance of HeV genome in different tissues for the HeV Redlands dose titration. The values obtained for the tissue from the occipital lobe of the brain are used as the calibrator, and quantification is relative to this tissue type in each animal. Boxed areas below the axis represent tissues where relatively less genome was detected than in the occipital lobe of the brain animal numbers and tissue are indicated along the *x*-axis. (A) Adrenal gland, bladder, brain-olfactory node, heart, kidney and liver. (B) Lymph nodes-bronchial, lymph nodes-retro pharyngeal, lung-apical node, lung-diagphragmatic node, spleen and testes. Numbering along the axis indicates the challenge dose groups. Two ferrets per group were exposed to 50 $TCID_{50}$ (numbers 1 and 2), 500 $TCID_{50}$ (numbers 3 and 4), 5000 $TCID_{50}$ (numbers 5 and 6) or 50,000 $TCID_{50}$ (numbers 7 and 8).

(ferret 6-4) remained afebrile and clinically well throughout the study. The single low dose animal ferret 5-4 also remained afebrile but showed reduced play activity on day 9 pc, developed weakness and tremor and was euthanized on day 10 pc.

### 3.5. Virus isolation and genome detection

Virus isolation and genome detection are shown in Table 2. Viral genome was detected in most tissues and fluids from control ferrets and virus was isolated from a number of, but not all, genome positive samples. In contrast, HeV genome was not detected in any of the tissues from the HeVsG immunised animals, including those from ferret 5-4 which was euthanized at d10 pc. HeV genome was not detected in the body fluids of HeVsG immunised ferrets except in nasal washes taken at day 6, 8 and 10 pc from ferret 6-4, with $C_t$ values ranging from 35.2 to 37.2. Both of the unvaccinated control ferrets, 7-0 and 8-0, were euthanased at day 7 pc and genome was detected in the nasal washes of 8-0 at this time. Virus isolation from ferret 6-4 nasal washes was unsuccessful.

### 3.6. Post-challenge serology

Control ferrets 7-0 and 8-0 were negative for serum neutralising antibody at day 6 pc and at euthanasia (day 7 pc). No significant or consistent rise in antibody titre was detected post-challenge in any HeVsG immunised ferrets (Table 3).

### 3.7. Gross pathology, histopathology and immunohistochemistry

Similar gross and histopathological lesions were observed in control ferrets 7-0 and 8-0 to those found in animals used for development of the infection model described above (Fig. 1). All other ferrets apart from ferret 5-4 were grossly and histologically normal. In ferret 5-4, both kidneys were pale and enlarged and the stomach contained hemorrhagic fluid. Histologically, acute renal tubular necrosis was identified that was not attributable to HeV infection in this animal (Fig. 1). No HeV antigen was detected in any tissue of HeVsG immunised ferrets.

**Table 2**
Genome detection and virus isolation from HeVsG vaccinated and control HeV challenged ferrets.

| Ferret # | Eut (dpc) | TaqMan real time PCR | | | | | | Virus isolation | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Swabs Or | Re | Nasal wash | Blood | Urine | Tissues | Swabs Or | Re | Nasal wash | Blood | Urine | Tissues |
| 1-100 | 21 | – | – | – | – | – | – | – | | – | – | – | – |
| 2-100 | 21 | – | – | – | – | – | – | – | | – | – | – | – |
| 3-20 | 21 | – | – | – | – | – | – | – | | – | – | – | – |
| 4-20 | 21 | – | – | – | – | – | – | – | | – | – | – | – |
| 5-4 | 10 | – | – | – | – | – | – | – | | – | – | – | – |
| 6-4 | 21 | – | – | + d6,8,10 | – | – | – | – | | – | – | – | – |
| 7-0 | 7 | + d6,7 | + d7 | – | + d6,7 | + d7 | 13/13 +ve | – | – | – | – | – | 7/13 +ve |
| 8-0 | 7 | + d7 | + d7 | + d7 | + d6,7 | + d7 | 12/13 +ve | + d7 | – | – | – | + d7 | 5/13 +ve |

Ferret # also indicates dose of HeVsG in μg, Eut (dpc) indicates day post challenge that ferrets were euthanized. Swabs: Or = oral swabs, Re = rectal swabs. – indicates no viral genome detected/no virus isolated; + indicates viral genome detected/virus isolated, with the days on which genome or virus was detected. For tissues '+ve' indicates the number of positive tissues from the 13 tissues analysed.

**Table 3**
HeV serum neutralisation titres from HeVsG vaccinated and control ferrets post-challenge.

| Ferret # and μg dose HeVsG | HeV SNT titre | | | | |
|---|---|---|---|---|---|
| | Day 30 pv | Day 6 pc | Day 8 pc | Day 10 pc | Day 21 pc |
| 1-100 | 1:8192 | 1:2048 | 1:4096 | 1:1024 | 1:512 |
| 2-100 | 1:8192 | 1:1024 | 1:512 | 1:4096 | 1:1024 |
| 3-20 | 1:8192 | 1:512 | 1:512 | 1:128 | 1:2048 |
| 4-20 | 1:16384 | 1:4096 | 1:512 | 1:512 | 1:8192 |
| 5-4 | 1:8192 | 1:2048 | 1:512 | ND | 1:512 |
| 6-4 | 1:8192 | 1:1024 | 1:128 | 1:512 | 1:256 |
| 7-0 | <1:2 | <1:2 | ND | ND | <1:2 |
| 8-0 | <1:2 | <1:2 | ND | ND | <1:2 |

SNT = serum neutralisation titre, pv = post vaccination, pc = post challenge.

### 3.8. Measurement of antibody to HeV F glycoprotein in ferret serum

As an indirect, alternative measure of virus replication in vaccinated animals, antibody to the HeV F glycoprotein was measured in sera using a Luminex microsphere assay (Fig. 3). The only source of the F glycoprotein in this experiment is the live viral challenge at day 41 of the experiment. Prior to challenge ferrets are exposed to HeVsG alone (except ferrets 7-0 and 8-0) therefore MFI values obtained up to day 41 represent those we find in sera from ferrets with no exposure to F glycoprotein. At the time of euthanasia, three weeks after challenge, MFI values in all 8 ferrets had not risen above those obtained prior to challenge, indicating that we were not detecting antibody to the F glycoprotein. From this we inferred that the challenge virus had not replicated. Antibody to HeV F glycoprotein was not detected in the virus controls but these animals were euthanized at d7 pc, before detectable antibody could develop.

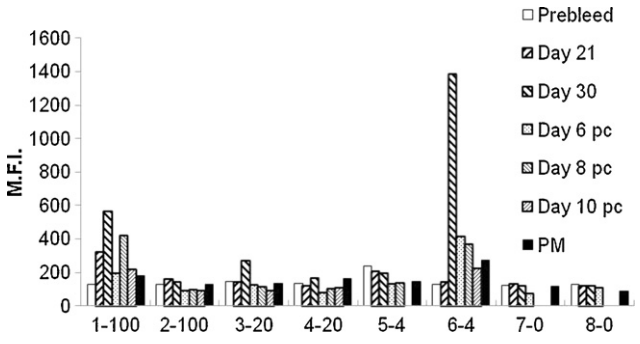


**Fig. 3.** Luminex assay showing no increase in detection of antibody to HeV F glycoprotein in serum after challenge with HeV at day 40. M.F.I. indicates mean fluorescence intensity. Ferret number on the *x*-axis also indicates dose of HeVsG in μg. PM (day of post mortem) varies. Both control ferrets (7-0 and 8-0) were euthanased at day 7 pc, ferret 5-4 was euthanased at day 10 pc. Remaining ferrets were all euthanased at d 21 pc.

## 4. Discussion

All known human cases of infection with HeV to date have resulted from close contact with infected horses, with no recorded instances of bat to human or human-to-human transmission [28]. These observations make the horse an attractive target for a HeV vaccination strategy to prevent virus shedding from infected horses, with the resulting interruption of the principal chain of transmission of HeV to humans preventing HeV disease in both. There are currently no licensed vaccines available for prevention of henipavirus disease. However, trials of various henipavirus vaccine candidates in three different animal models – cats, hamsters and pigs [22,25–27] indicate that a successful vaccination strategy against disease caused by HeV and NiV should be possible.

Here we assessed the suitability of the ferret as a model for (i) infection with HeV and the resultant virus-induced disease and (ii) vaccination against the disease caused by HeV. Ferrets fulfil some important requirements for working with a BSL-4 pathogen. Compared to hamsters they are large enough to allow more sophisticated sampling interventions to be carried out on individual animals over the time course of an infection, they are easier to handle than pigs and cats, and develop disease much more reliably than pigs. Ferrets have been successfully used to model other human respiratory infections such as influenza [34] and SARS [35] as well as NiV [21,29]. Similarly to NiV, exposure of ferrets to a relatively low challenge dose of HeV consistently results in all animals developing an acute, fulminating systemic infection characterised by wide-spread vasculitis and affecting multiple major organ systems particularly the lung and central nervous system.

Using this ferret model of HeV infection and pathogenesis we evaluated an immunization strategy with a subunit vaccine based on recombinant HeVsG. As observed in a feline model of NiV challenge [27], a 100 μg dose of HeVsG successfully prevented clinical disease with no evidence of viral replication or shedding as detected by TaqMan real-time PCR, virus isolation, histology or immunohistology. In addition, virus infection, replication and shedding was also prevented in ferrets in the 20 μg and 4 μg dosing groups of the HeVsG immunogen, with the exception of one animal in the low dose group. Here, genome was detected in the nasal washes of this animal (ferret 6-4) at three consecutive sampling times up to day 10 pc. Importantly, as viral genome was not detected in the nasal washes of any other ferret this is consistent with viral replication rather than detection of the original inoculum. No HeV genome was detected in any other tissue or body fluid of this ferret, nor did the ferret exhibit any signs of illness, indicating that the immune response generated by the 4 μg vaccine dose was sufficient to limit infection to the primary site of exposure. Prevention of virus replication was also supported by the lack of an anamnestic antibody response to the virus challenge as well as the absence of an antibody response to HeV F glycoprotein in any of the vaccinated ferrets 21 days after challenge.

In a similar experiment, cats immunised with 5, 25 and 50 μg HeVsG vaccine and challenged with NiV showed evidence of viral replication with increasing antibody titres post challenge and genome detection in oral swabs, urine and the brains of 4 animals receiving two higher doses of vaccine [26]. The detection of viral genome in the brain of cats with significant antibody levels prior to challenge indicated that a persistent infection might occur despite pre-existing immunity. A farmer infected during the first recorded outbreak of HeV that occurred in Mackay in August 1994 recovered from meningitis only to develop neurologic signs 14 months later and die with HeV present in the brain [36].

Variation in vaccination regimes, adjuvants used, the challenge virus stock and dose do not allow for an absolute direct comparison between any of the recombinant HeVsG vaccination trials carried out to date. However, these various trials clearly indicate that vaccination with HeVsG can prevent clinical HeV disease, and in some cases HeV infection, depending on the trial parameters as seen in at least two animal models. Prevention of infection is vital for preventing the establishment of a persistent infection from which virus could recrudesce some time after recovery. Also of similar importance, especially in the case of a vaccination scenario for horses, the prevention of infectious virus shedding could be considered a critical goal, and our findings here indicate that the higher doses of at least 20 μg was able to meet this standard. Future studies are planned to assess the recombinant HeVsG vaccination strategy in ferrets further, as well as assess the performance of the vaccine in non-human primates and in horses.

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