Chloroquine administration does not prevent Nipah virus infection and disease in ferrets

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Abstract

Hendra virus and Nipah virus, two zoonotic paramyxoviruses in the genus Henipavirus, have recently emerged and continue to cause sporadic disease outbreaks in humans and animals. Mortality rates of up to 75% have been reported in humans, but there are presently no clinically licensed therapeutics for treating henipavirus-induced disease. A recent report indicated that chloroquine, used in malaria therapy for over 70 years, prevented infection with Nipah virus in vitro. Chloroquine was assessed using a ferret model of lethal Nipah virus infection and found to be ineffective against Nipah virus infection in vivo.
Hendra virus (HeV) and Nipah virus (NiV) are the sole members of the genus Henipavirus (8, 23), for which the natural reservoir is several species of flying foxes. NiV was first isolated during an outbreak of disease in humans and pigs in Malaysia in 1998 (5). The virus was transmitted from pigs to humans and was associated with a high level of mortality - 265 human cases with 105 deaths. Subsequently, multiple NiV outbreaks have been reported in people in Bangladesh and India, with respiratory as well as neurological symptoms recorded and mortality rates of up to 75% observed. There was also strong epidemiological evidence of human-to-human transmission in at least one of these outbreaks (1, 11). HeV was first isolated in Queensland, Australia in 1994 where it led to the death of 13 horses and one horse trainer (16). Eleven subsequent spill-over events resulted in four human infections including two fatalities (9), with both respiratory and neurological symptoms recorded. In each case, transmission of HeV to humans appears to have been via infected horses rather than from contact with natural reservoirs (10, 24).

Together, the henipaviruses present a continued potential threat to people in close contact with infected animals, and human-to-human transmission remains a possibility. HeV and NiV are classified as select agents with the potential for causing significant morbidity and mortality in humans, and major economic and public health impacts. Therefore work with live virus requires Biosafety Level 4 (BSL-4) containment. There are currently no prophylactic or therapeutic treatments available. Empirical success was reported with ribavirin during one outbreak of NiV, with a 36% reduction in mortality observed (4); however the trial was not blinded or randomised. Although monoclonal antibodies and subunit vaccine candidates are currently under development (2), none are yet licensed for use in humans.
Chloroquine is a 9-aminoquinoline used widely in the mid 20th century as an anti-malarial agent until resistance emerged in the malaria parasite. A recent report showed that chloroquine at a concentration of 1 µM or greater inhibited infection with live HeV and NiV in vitro (20). In this study we report on the efficacy of chloroquine in preventing or moderating infection and disease in vivo in a ferret model of NiV (2) (K. N. Bossart, Z. Zhu, D. Middleton et al., submitted for publication).

Ferrets aged 12 to 18 months received 25 mg/kg/day of chloroquine (Sigma Aldrich, NSW, Australia) in 500 µl of 20% sucrose. This dose was chosen based on a previous study of chloroquine treatment in humans suffering from acute malaria (3), and is twice the dose used in a study to determine the effect of chloroquine on influenza infection in ferrets (22). Ferrets 1-3 received a loading dose of chloroquine 24 hours before viral challenge and ferrets 4-6 received their first dose of chloroquine 10 hours after viral challenge. Control ferrets 7 and 8 were given a mock dose of 20% sucrose; one at 24 hours before and one at 10 hours after challenge. The challenge virus used was a low passage of the NiV Malaysia isolate EUKK 19817 (13) administered oronasally at 5,000 TCID\textsubscript{50} per animal (~ 10x MID\textsubscript{50}). All procedures involving live virus were conducted under BSL-4 containment.

Daily clinical observations were recorded and body temperature assessed continuously via implanted temperature transponders. Nasal washes, rectal and oral swabs, urine, serum and EDTA blood samples were collected from animals anaesthetised with ketamine (Ketamil, Troy Laboratories, NSW, Australia) and medetomidine (Domitor, Pfizer Animal Health, NSW, Australia). Samples were
collected prior to the first administration of chloroquine, at days 6 and 8 post
challenge, and at euthanasia. At post mortem examination, the tissues listed in Table
were collected for virus isolation, viral genome detection, histology and
immunohistology.

Swabs were collected into 1 ml of PBS. Tissue samples were either collected in 1 ml
of PBS for virus isolation, submerged in 800 µl MagMAX buffer (Ambion, VIC,
Australia) for genome extraction, or fixed in 10% neutral buffered formalin for 48 h
prior to routine processing for histology. RNA was extracted using the MagMAX–96
Viral RNA Isolation Kit (Ambion). TaqMan real time PCR was carried out using the
AgPath-ID™ One-Step RT-PCR Kit (Applied Biosystems, VIC, Australia) targeting
the N gene of NiV using primers, FOR- (5’- TCAGCAGGAAGGCAAGAGA TA-3’), REV- (5’- CCCTTCATCGATATCTTGATCA-3’), and probe- (5’-6FAM-
CCTCCAATGAGCACACCTCAG-TAMRA-3’). Positive results were defined
by a cycle threshold (Ct) value of ≤ 40. For virus isolation, supernatants from
homogenised tissues positive for NiV genome were incubated on Vero cell
monolayers and scored positive if syncytia were present after six days.

Ferrets were euthanased if they reached a pre–determined humane endpoint based on
experience with NiV infection in cats (14, 15). Endpoints were a surrogate for
lethality and included fever plus signs of rapidly progressing clinical illness. All
ferrets were febrile (>40º C) by day 6 pi and deteriorated rapidly thereafter.
Euthanasia was carried out on day 7 or 8 pi with ferrets severely depressed or
obtundated, and showing various combinations of hind limb paresis, tremor,
myoclonus, urinary incontinence, subcutaneous edema of the neck and throat,
petechial hemorrhages in the skin and blood in oral secretions. There were no clinical differences noted between treatment and control animals. Similarly, at post mortem examination, abnormalities detected included pin-point hemorrhagic nodules throughout the lung, enlarged hemorrhagic and edematous sub-mandibular and retropharyngeal lymph nodes, and petechial hemorrhages of viscera. Histological examination confirmed systemic vasculitis in each ferret, with prominent endothelial syncytia in spleen, kidney, lung, lymph node and meninges. Severe bronchointerstitial pneumonia and glomerulonephritis with syncytial formation were also identified. No differences were observed in lesion severity or viral antigen load (assessed using a rabbit polyclonal antiserum against recombinant NiV nucleoprotein) between treatment and control animals (Fig. 1).

Viral RNA was detected in all fluids and tissues from each ferret. 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 C_T method (12). Viral RNA was detected most abundantly in the retropharyngeal lymph node, apical node of the lung, spleen, reproductive organs and adrenal gland (Table 1). These tissues were also amongst those from which virus was most consistently isolated (Table 1). Further, virus distribution in vivo did not vary between treatment and control animals apart from the additional abundant virus detection and isolation from the heart of one control ferret (#8).

Relative quantification of viral RNA levels in selected tissues between ferrets was carried out relative to a control ferret (#7). NiV RNA distribution was random with treated ferrets sometimes showing higher relative RNA levels than control animals.
and vice versa (Fig. 2). Nevertheless, the failure to show consistently higher levels of
viral RNA in control compared with treated animals indicated that virus replication
had not been reduced in animals receiving chloroquine.

As the pharmacokinetics of chloroquine in ferrets is unknown, we used mass
spectrometry to estimate chloroquine concentrations in serum. Based on a published
method (21), aliquots of serum (20 µl) were precipitated with methanol (180 µl),
vortexed for 20 sec and centrifuged at 12,000 g for 10 min. A 100 µl aliquot of
supernatant was diluted 1:1 with 0.4% v/v formic acid to give final solvent
composition 50% methanol/ 0.2% formic acid. Diluted samples were directly infused
into an LCQ ion-trap mass spectrometer (Thermo, San Jose, CA). Data collected from
selected reaction monitoring (SRM) using the precursor → product ion transition of
m/z 320 → 247 was used to estimate chloroquine in serum samples. Serum was taken
from all 8 ferrets prior to any treatment, pooled, spiked with chloroquine, and then
used to generate a standard curve from which chloroquine concentrations in the serum
of experimental animals at euthanasia were estimated. While no chloroquine was
detected in the serum of the two control ferrets, the six ferrets that received the drug
had estimated serum concentrations ranging from 1.6 – 16.8 µM; at least equivalent to
the reported effective in vitro dose of 1 µM (20) and also within a similar plasma
concentration range (1.6-12.5 µM) detected in humans receiving chloroquine
treatment (7).

To confirm that chloroquine was active against NiV infection in our hands we carried
out an in vitro assay based on that of Porotto et al (20). Chloroquine at 0.4, 0.2 and 0.1
µM prevented the spread of NiV infection beyond single cells, with no syncytia
Viral spread via F-mediated cell-cell fusion requires that the henipavirus F protein is cleaved to an active form by Cathepsin L in the endosomes, and this cleavage is pH dependent (6, 17, 18, 19). Reduced henipavirus spread observed in the presence of chloroquine led to speculation that chloroquine prevents proteolytic cleavage of henipavirus F – either by directly inhibiting Cathepsin L or by an effect on endosomal pH and without this step virions are not infectious (20). Obviously, there is a difference between the in vivo and the in vitro results achieved with chloroquine treatment of NiV infection, but the reason for the discrepancy is unknown. However, a similar outcome has been reported for influenza treatment with chloroquine (22).

Although chloroquine was effective in preventing the spread of NiV infection in vitro, it did not prevent the spread of NiV infection in vivo when used either as a prophylactic or a post-exposure therapeutic. By a range of measures employed in the present study, NiV replication and pathology were unaltered between the treatment and control groups of animals. We confirmed that chloroquine was active against NiV in vitro and established that all ferrets at the time of euthanasia had estimated serum concentrations of chloroquine greater than that required for anti NiV activity in vitro. From these observations we conclude that chloroquine is not likely to be a useful drug in the treatment of henipavirus infections.

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References


**Figure legends**

**Figure 1.** Immunohistochemical detection of NiV antigen in lung tissue from a ferret treated with chloroquine after NiV challenge (A) or before NiV challenge (A-inset) compared to a control ferret (B). Bar = 50 µm.

**Figure 2.** Relative quantification of NiV RNA in selected tissues between different ferrets. Quantification is relative to control ferret #7 (labelled). For each tissue the first 6 bars (left to right) represent relative NiV RNA levels in ferrets #1-3 (treated with chloroquine 24 h pre-challenge) and #4-6 (treated with chloroquine 10 h post challenge). The last 2 bars represent control ferrets #7-8 (no chloroquine).
Table 1. Relative quantification of NiV RNA between different tissues in each animal. The occipital lobe of the brain (shaded) is used as the calibrator and quantification is relative to this tissue type in each animal. Tissues that were also positive by virus isolation are indicated in bold. N/A, results for sex organs for half of the animals only. The gender of each animal is indicated by the single letter code in brackets after the animal number (F = female and M = male).

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<td>Treated with chloroquine 10 hours after NiV challenge</td>
<td>Untreated control, NiV challenge only</td>
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Relative NiV RNA levels

treated ferrets, 1-6
untreated ferrets, 7-8

occipital lobe
olfactory pole
kidney
lung - apical node
lung diaphragmatic node

0 2 4 6 8 10 12