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Assessing the development of oseltamivir and zanamivir resistance in A(H5N1) influenza viruses using a ferret model

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ABSTRACT

Using an *in vivo* ferret model, we investigated the development of resistance to oseltamivir and zanamivir for two different influenza A(H5N1) viruses (A/Vietnam/1203/2004, haemagglutinin phylogenetic clade 1, and A/Chicken/Laos/26/2006, haemagglutinin phylogenetic clade 2.3) by treating the animals with doses equivalent either to the recommended human treatment dose or a range of sub-optimal drug doses. No resistance was observed in oseltamivir-treated ferrets, but analysis of nasal washes from zanamivir-treated ferrets infected with influenza A/Vietnam/1203/2004 revealed one viral isolate (from a ferret receiving the highest dose of zanamivir, 1.0 mg/kg twice daily) with a zanamivir IC_{50} that was 350-fold higher than the other isolates tested. The same virus also demonstrated a 26-fold increase in oseltamivir IC_{50}. The isolate with reduced susceptibility was taken from a ferret 8 days post-infection that was being treated with the recommended human zanamivir dose. Sequence analysis of the resistant virus revealed a glutamine (Q) to leucine (L) mutation at residue 136 of the neuraminidase. This is the first report of this mutation being associated with neuraminidase inhibitor susceptibility and one of the few reported mutations that confer zanamivir resistance, and as such should be closely monitored in influenza A(H5N1) and other N1 viruses in the future. Further animal studies and human clinical trials are necessary to optimize neuraminidase inhibitor dosing strategies for the treatment of influenza A(H5N1) infections.

1. Introduction

Highly pathogenic A(H5N1) avian influenza viruses have infected poultry throughout parts of Asia, Middle East, Europe and Africa, resulting in the death or culling of many animals and significant economic loss in affected countries (Webster and Govorkova, 2006). On occasions, humans have also become infected with the A(H5N1) virus with the vast majority of infections having been caused by close contact with infected poultry (Abdel-Ghafar et al., 2008). Viruses circulating in different regions are antigenically and genetically distinct and have been classified into at least 10 separate phylogenetic clades and subclades (Abdel-Ghafar et al., 2008). A(H5N1) infections in humans typically cause severe pneumonia, which often progresses to acute respiratory distress syndrome, and on occasions can cause gastrointestinal symptoms, leukopenia and lymphopenia (de Jong et al., 2006). A(H5N1) infection has also been associated with a disseminated spread of the virus throughout the body, with infectious virus being detected in the blood and cerebrospinal fluid of some severely ill patients (de Jong et al., 2005b). As a result of the highly pathogenic nature of the virus, case fatality rates have been high. Over 490 confirmed human cases of A(H5N1) infection have occurred worldwide since 2003, resulting in 292 deaths. This gives an overall case fatality rate (CFR) of approximately 60% although this varies considerably between countries. Indonesia, where most cases of A(H5N1) infection have been reported, has a significantly higher CFR of 82% compared to the CFR for cases seen in Egypt of 31% (WHO, 2009).

Although human-to-human transmission of the virus has been rare to date, the potential for mutation of the A(H5N1) virus to facilitate transmission and rapid spread throughout the human population, leading to a pandemic, is still a possibility. Although A(H5N1) vaccine clinical trials have yielded promising results, they are not publically available for use, and therefore antiviral drugs remain the only immediately available measure for the control of A(H5N1) infections. The neuraminidase inhibitors (NAIs), oseltamivir (Tamiflu) and zanamivir (Relenza) are currently the most appropriate options for the treatment of A(H5N1) infection. Of these, oseltamivir has been the most widely used and has been demonstrated to improve patient survival, although early administration of the drug significantly improves its effectiveness

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(Abdel-Ghafar et al., 2008; de Jong and Hien, 2006). Previous animal studies have suggested that the oseltamivir dose and duration of treatment may need to be increased for A(H5N1) viruses compared to recommendations for seasonal influenza (Boltz et al., 2008; Govorkova et al., 2007; Yen et al., 2005). There is currently little data on the effectiveness of zanamivir treatment for prophylaxis of A(H5N1) infection in humans or in animal models.

The effectiveness of any antiviral drug can be significantly impaired if a virus develops resistance. Oseltamivir resistant A(H5N1) viruses containing a H274Y mutation (N2 numbering) in the neuraminidase (NA) gene have been detected in two patients undergoing oseltamivir treatment (de Jong et al., 2005a; Le et al., 2005), and may have been associated with the clinical deterioration and fatal outcomes in these individuals. We have previously conducted *in vitro* studies that selected for drug resistant variants during cell culture passage of A(H5N1) virus in increasing NAI concentrations (Hurt et al., 2009a). In that study we identified a range of mutations that can occur in A(H5N1) viruses under oseltamivir or zanamivir pressure, although *in vitro* experiments may not accurately predict the likelihood of these resistant strains occurring in humans during drug treatment. Here we use an *in vivo* ferret model to investigate the development of resistance to oseltamivir and zanamivir in A(H5N1) viruses by treating the animals with doses either equivalent to the normal human treatment dose (Ward et al., 2005) or a range of sub-optimal drug concentrations. Analysis of viruses shed by the NAI treated ferrets detected one zanamivir-resistant isolate that contained a novel glutamine (Q) to leucine (L) mutation at residue 136, an amino acid that has recently been demonstrated to play a role in zanamivir susceptibility following the detection of a Q to lysine (K) mutation at the same residue in cultured seasonal A(H1N1) viruses (Hurt et al., 2009c; Okomo-Adhiambo et al., 2010).

2. Materials and methods

2.1. Viruses

Two A(H5N1) influenza viruses classified as Highly Pathogenic Notifiable Avian Influenza according to World Organisation for Animal Health (OIE) criteria, namely A/Vietnam/1203/2004 (Vn/1203) (haemagglutinin (HA) phylogenetic clade 1) and A/Chicken/Laos/26/2006 (Laos/26) (HA phylogenetic clade 2.3) (Abdel-Ghafar et al., 2008) (kindly supplied by Paul Selleck, Australian Animal Health Laboratory, Australia), were cultured in embryonated eggs to titres of $1 \times 10^{8.33}$ EID_{50}/0.1 ml and $1 \times 10^{8.70}$ EID_{50}/0.1 ml respectively in enhanced BSL3 conditions at the CSIRO Australian Animal Health Laboratory, Australia.

2.2. NAIs and drug administration in ferrets

The prodrug oseltamivir phosphate was kindly provided by Hoffmann-La Roche Ltd., Switzerland, and zanamivir was kindly provided by GlaxoSmithKline, Australia. A range of dilutions of oseltamivir phosphate were prepared in 1 ml sugar solutions of sterile PBS (15 g/100 ml) and given in four different dose regimes: 0.1, 0.5, 2.5 and 5.0 mg/kg delivered orally 24 and 2 h pre-infection, and then 5 h post-infection, followed by twice daily (12 h apart) for a further 10 days. Zanamivir dilutions were prepared in 0.2 ml doses of sterile PBS and given in four different dose regimes: 0.02, 0.1, 0.5 and 1.0 mg/kg and delivered intranasally 24 and 2 h pre-infection, and then 5 h post-infection followed by twice daily (12 h apart) for a further 10 days. Due to the high levels of mortality expected with the A(H5N1) viruses being used in this study, a prophylactic regime, rather than a therapeutic regime, was chosen to increase the period of time that ferrets would remain alive and therefore permit the greatest opportunity for NAI resistant viruses to emerge. Control ferrets not under drug treatment received either a sugary solution of sterile PBS delivered orally, or sterile PBS delivered intranasally, at the same time intervals as the ferrets under drug treatment.

2.3. Ferrets and nasal washing

Female ferrets aged approximately 6 months and weighing approximately 1 kg were sourced from Institute of Medical and Veterinary Science, South Australia, and were tested and found to have no detectable pre-existing antibodies to recent human A(H1N1), A(H3N2) or influenza B viruses, or to highly pathogenic avian A(H5N1) viruses, by haemagglutination-inhibition assay. Two separate ferret experiments were performed, in the first experiment ferrets were infected with the Vn/1203 strain, whilst the second experiment involved the infection of ferrets with the Laos/26 virus. Each of these experiments involved a total of 18 ferrets that were housed in pairs across nine separate cages. One pair of ferrets was used as a control and animals received no drug treatment. Four pairs of ferrets were subjected to zanamivir treatment, with each pair receiving either 0.02, 0.1, 0.5 or 1.0 mg/kg doses of zanamivir (i.e. two ferrets per drug concentration). Similarly the remaining four pairs of ferrets were subjected to oseltamivir treatment, with each pair receiving either 0.1, 0.5, 2.5 or 5.0 mg/kg doses of oseltamivir. Ferrets were not under anaesthesia during either zanamivir or oseltamivir dosing; 500 μl of virus containing 10^6 egg infectious doses (EID_{50}) was used to intranasally infect each ferret whilst under ketamine/medetomidine anesthesia (50:50, 0.1 ml/kg, reversed with atipemazole). Nasal washes were taken from anesthetised ferrets using an Optiva (Medex) 25 gauge i.v. catheter and 1 ml of PBS containing 1% (w/v) BSA, 100 μg/ml streptomycin and 100 U/ml penicillin at least 6 h after any drug treatment dose to avoid contaminating the nasal wash with residual drug. Nasal washes were stored at −70 °C. Virus was cultured in embryonated chicken eggs at 37 °C for 48 h to determine the presence or absence of infectious virus and then, if virus was present, tested for drug susceptibility. Egg culture was shown to have superior sensitivity compared to MDCK cell culture, with the egg culture limit of detection being 0.1 cell culture infectious $dose_{50}$ ($CCID_{50}$) for the Laos/26 strain and 0.3 $CCID_{50}$ for the Vn/1203 virus. Animals that showed signs of severe disease, central nervous system involvement, or >20% weight loss within 7 days of challenge were euthanized according to predetermined humane endpoints. Surviving ferrets were humanely killed on day 14. At the time of death, samples from lung, spleen, hind brain, fore brain, kidney, liver, pancreas, heart and small intestine were taken from each ferret, homogenised and virus culture attempted in embryonated chicken eggs. Ferret studies were approved by the CSIRO Australian Animal Health Laboratory Animal Ethics Committee.

2.4. Serology

Serum samples were collected at the time of death, treated with receptor-destroying enzyme (Denka Seiken, Japan) according to the Manufacturer's instructions at 37 °C overnight, heat inactivated at 56 °C for 30 min, and tested by haemagglutination-inhibition assay with homologous reference A(H5N1) antigen and antiserum using 1% chicken red blood cells.

2.5. NA inhibition assay

A fluorescence-based NA enzyme inhibition assay was used to test cultured viruses for their susceptibility to either zanamivir or oseltamivir. Oseltamivir carboxylate, the active form of the ethyl ester prodrug oseltamivir phosphate, was used for testing the susceptibility of viruses in the NA inhibition assay and was

Table 1
Pathogenicity and viral shedding of A/Vietnam/1203/2004 A(H5N1) virus in ferrets.

Ferret #	Oseltamivir dose (mg/kg) twice daily	Nasal wash collected on indicated day post-virus inoculation[a]						Day of death	Seroconversion[b]
		2	4	6	8	10	14		
A									
10	None	+	+	+	NS	NS	NS	Day 7	<4
11	0.1	–	+	NS	NS	NS	NS	Day 4	<4
12	0.1	+	NS	NS	NS	NS	NS	Day 4	NS
13	0.5	+	NS	NS	NS	NS	NS	Day 2	<4
14	0.5	+	+	NS	NS	NS	NS	Day 4	<4
15	2.5	+	+	+	NS	NS	NS	Day 6	4
16	2.5	+	+	+	–	NS	NS	Day 6	8
17	5.0	+	+	+	+	–	–	Survived	16
18	5.0	–	–	–	–	–	–	Survived	16
Ferret #	Zanamivir dose (mg/kg) twice daily	Nasal wash collected on indicated day post-virus inoculation[a]						Day of death	Seroconversion[b]
		2	4	6	8	10	14		
B									
1	None	+	+	NS	NS	NS	NS	Day 4	<4
2	0.02	+	+	NS	NS	NS	NS	Day 6	NS
3	0.02	+	+	+	+	+	NS	Day 10	8
4	0.1	+	+	NS	NS	NS	NS	Day 4	<4
5	0.1	+	+	NS	NS	NS	NS	Day 4	<4
6	0.5	+	+	+	+	–	–	Survived	64
7	0.5	+	+	+	NS	NS	NS	Day 7	<4
8	1.0	+	+	NS	NS	NS	NS	Day 4	<4
9	1.0	+	+	+	(+)	NS	NS	Day 8	<4

Effectiveness of four concentrations of (A) oseltamivir and (B) zanamivir in reducing mortality and duration of viral shedding.

[a] '+' indicates the detection of infectious virus in nasal wash (as determined following egg culture and haemagglutination assay); '(+)' indicates the detection of infectious virus with raised IC_{50} in nasal wash; '–' indicates that no infectious virus was detected; 'NS' indicates that no sample was available for testing due to death or euthanasia.

[b] Seroconversion was tested at day 14 post-inoculation using an HI with homologous reference A(H5N1) antigen and antiserum. All prebleeds had an HI titre of <4.

kindly provided by Hoffmann-La Roche Ltd., Switzerland. Viruses were diluted to an equivalent NA activity and mixed with varying concentrations of NAI in microtitre plates (FluoroNunc plates, Nunc). Final reaction mixture concentrations of the NAIs ranged from 0.01 to 10,000 nM. The virus/inhibitor mix was incubated at room temperature for 45 min prior to the addition of 50 μl of 2-(4-methylumbelliferyl)-a-D-*N*-acetylneuraminic acid (MUNANA) substrate (Sigma–Aldrich) (0.3 mM) and then incubated at 37 °C for 60 min. The reaction was terminated by the addition of 100 μl of stop solution (0.824 M NaOH in absolute ethanol) (Hurt et al., 2004). The data were plotted as the percentage of fluorescence activity inhibited against the log NA inhibitor concentration. The concentrations required to inhibit 50% of NA activity (IC_{50} values) were calculated using the logistic curve fit program "Robosage" kindly provided by GSK, UK. Control susceptible and resistant strains were included in each assay. Viruses with IC_{50} values greater than the mean ± 1 standard deviation of A(H5N1)-susceptible viruses were sequenced.

2.6. RT-PCR and sequencing

Viral RNA was extracted from 200 μl of either egg allantoic fluid or ferret nasal wash solution using the MagnaPure extraction system, according to the Manufacturer's recommendations (Roche, Australia) with an elution volume of 90 μl. A 5 μl aliquot of RNA was used to amplify the selected influenza gene using specific primers (sequences available on request) and the SuperScriptIII Platinum® One-step RT-PCR System (Invitrogen) reagents. Amplicons were visualized on a 2% agarose gel. PCR products were purified for use in a sequencing reaction using the QIAquick PCR Purification Kit (Qiagen). DNA sequencing was carried out using the ABI Prism Dye Terminator III cycle sequencing Kit (Applied Biosystems) followed by the removal of excess dye terminators using a DyeEx Spin Kit (Qiagen). The sequence was determined using an automated capillary DNA sequencer (ABI Prism 377 located at the Institute of Medical and Veterinary Science, Adelaide, Australia). Sequences were assembled and aligned using the DNAStar Lasergene 8 package.

3. Results

3.1. Effect of NA inhibitor treatment on survival and duration of viral shedding

Two different A(H5N1) influenza viruses, Vn/1203 and Laos/26, were used in this study to infect ferrets and to determine how readily resistance was generated under NAI selective pressure. Although both viruses fulfilled the OIE criteria for Highly Pathogenic Notifiable Avian Influenza in chickens they were found to cause significantly different morbidity in ferrets. Control ferrets that were not treated with either NAI and were infected with Vn/1203 showed severe disease and were euthanized on days 4 and 7 post-infection on humane grounds; whereas control ferrets infected with Laos/26 survived until the end of the experiment (Tables 1 and 2) having shown comparatively few signs of disease (data not shown). The highly pathogenic nature of the Vn/1203 strain was also evident in the ferrets under NAI treatment. Only the highest oseltamivir dose of 5 mg/kg twice daily prevented death or euthanasia on humane grounds following infection with the Vn/1203 strain; all ferrets receiving lower doses reached predetermined humane endpoints and were euthanized between days 2 and 6 (Table 1A). Analysis of this data using Fisher's exact test (one-sided) confirmed that an oseltamivir dose of 5.0 mg/kg twice daily had a significant benefit on survival ($p = 0.028$) compared to any lower dose of the drug. As well as preventing mortality in both ferrets, the 5 mg/kg twice daily oseltamivir dose also prevented any detectable viral shedding in one of the ferrets inoculated with Vn/1203. High mortality was also observed in Vn/1203 infected ferrets under zanamivir treatment, with all but one ferret euthanized during the course of the experiment (Table 1B). The one animal that survived was treated with 0.5 mg/kg of zanamivir twice daily, even though animals treated with twice that concentration (1.0 mg/kg twice daily)

Table 2
Pathogenicity and viral shedding of A/Chicken/Laos/26/2006 A(H5N1) virus in ferrets.

Ferret #	Oseltamivir dose (mg/kg) twice daily	Nasal wash collected on indicated day post-virus inoculation[a]						Day of death	Seroconversion[b]
		2	4	6	8	10	14		
A									
36	None	+	+	+	–	–	–	Survived	8
27	0.1	+	+	+	–	–	–	Survived	8
28	0.1	+	+	+	–	–	–	Survived	32
29	0.5	+	+	+	–	–	–	Survived	8
30	0.5	+	+	+	+	–	–	Survived	8
31	2.5	+	+	+	–	–	–	Survived	16
32	2.5	–	–	–	–	–	–	Survived	4
33	5.0	+	–	–	–	–	–	Survived	8
34	5.0	+	+	+	–	–	–	Survived	8
Ferret #	Zanamivir dose (mg/kg) twice daily	Nasal wash collected on indicated day post-virus inoculation[a]						Day of death	Seroconversion[b]
		2	4	6	8	10	14		
B									
35	None	+	+	+	–	–	–	Survived	16
19	0.02	+	+	+	–	–	–	Survived	32
20	0.02	–	+	+	–	–	–	Survived	4
21	0.1	+	+	–	–	–	–	Survived	8
22	0.1	–	–	–	–	–	–	Survived	16
23	0.5	+	+	+	–	–	–	Survived	8
24	0.5	–	–	–	–	–	–	Survived	8
25	1.0	+	+	+	–	–	–	Survived	8
26	1.0	+	+	–	–	–	–	Survived	8

Effectiveness of four concentrations of (A) oseltamivir and (B) zanamivir in reducing mortality and duration of viral shedding.

[a] '+' indicates the detection of infectious virus in nasal wash (as determined following egg culture and haemagglutination assay); '–' indicates that no infectious virus was detected.

[b] Seroconversion was tested at day 14 post-inoculation using an HI with homologous reference A(H5N1) antigen and antiserum. All prebleeds had an HI titre of <4.

were both euthanized. When analyzed using Fisher's exact test there was no statistically significant survival benefit ($p = 0.44$) from a dose of ≥0.5 mg/kg zanamivir twice daily compared to lower doses. In contrast, all of the animals infected with the Laos/26 virus survived, including those that received low or even no antiviral treatment (Table 2). Viral shedding was detected up to day 6 in both control ferrets infected with this virus. Oseltamivir treatment had no affect on reducing the duration of viral shedding in the majority of animals, although in two animals (treated with 2.5 and 5.0 mg/kg twice daily) virus was either not detected at all, or was detected only on day 2 (Table 2A), although this was not statistically significant ($p = 0.167$). There was also no statistically significant dose-specific reduction in duration of viral shedding due to zanamivir treatment ($p = 0.119$), even though there was no detectable viral shedding in two ferrets treated with 0.1 and 0.5 mg/kg twice daily. Lack of statistical significance may be due to the low number of ferrets analyzed for this part of the study. As such, from the limited number of ferrets used in the experiments it appears that at the highest oseltamivir dose (5.0 mg/kg twice daily) there were significant survival benefits, but this was not observed with the highest zanamivir dose tested, and neither of the drugs had a significant impact on the duration of viral shedding in animals infected with the less pathogenic Laos/26 virus.

3.2. Serology

The three ferrets that survived the Vn/1203 infection developed antibody titers to the virus confirming infection had occurred, whereas many animals that were euthanized or died during the experiment did so prior to the production of any detectable antibodies (Table 1). Antibody was detectable in all of the ferrets infected with the Laos/26 virus even in those under NAI treatment. Antibody titers were generally low and there was no trend between antibody titer and drug dose. In addition, HI antibody titers of 4, 16 and 8 were detected in ferrets #32, #22, #24 respectively, that were found not to be shedding detectable levels of Laos/26 virus, confirming that these animals did become infected and seroconverted, but presumably were shedding virus at titers below the detection level of egg culture.

3.3. NA inhibitor susceptibility of viruses

All influenza positive isolates were tested for oseltamivir and zanamivir susceptibility in a fluorescence-based enzyme inhibition assay. Of these, one isolate demonstrated a significantly raised zanamivir IC_{50} (218.9 ± 19.6 nM; mean IC_{50} ± standard deviation) that was approximately 350-fold higher than the other A(H5N1) isolates tested (0.6 ± 0.1 nM; mean IC_{50} ± standard deviation) (Table 3). The same isolate also demonstrated a more moderate 26-fold increase in oseltamivir IC_{50} (Table 3). The isolate with raised IC_{50} values was grown from a day 8 nasal wash from ferret #9, that was infected with Vn/1203 and was being treated with the highest dose of zanamivir (1.0 mg/kg twice daily) (Table 1). After collection of the nasal wash from ferret #9 on day 8, the animal was euthanized. Virus was isolated from the fore brain and hind brain, but not from other organs, and direct RT-PCR of the organs was able

Table 3
NA inhibitor susceptibility of influenza viruses isolated from ferret nasal washes.

Virus[a]	Mean IC_{50} ± standard deviation (nM)	
	Zanamivir	Oseltamivir
All susceptible A(H5N1) viruses ($n = 84$)	0.6 ± 0.2	0.5 ± 0.4
Ferret #9/day 8 nasal wash	**218.9 ± 19.6**	**7.9 ± 2.1**
Ferret #9/day 8 fore brain	0.8 ± 0.1	0.2 ± 0.07
Ferret #9/day 8 hind brain	0.5 ± 0.1	0.1 ± 0.06

The isolate listed in bold contains the Q136L NA mutation.

[a] All viruses were egg grown isolates at passage level E1.

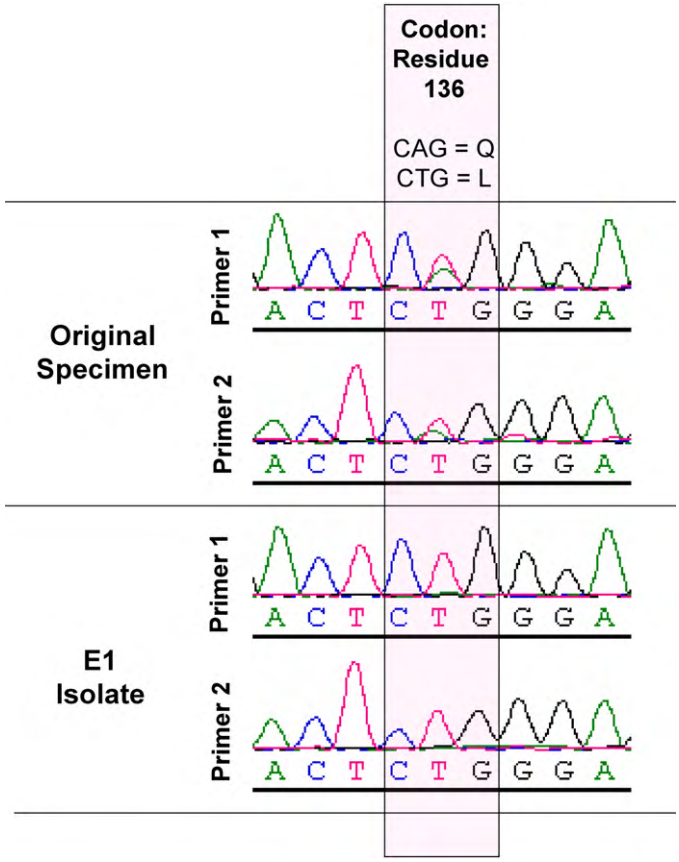

Fig. 1. Sequence chromatogram for the original specimen shows a mixed population of virus, some with the codon CAG (translates to leucine (L) residue) and some with codon CTG (translates to glutamine (Q) residue) at position 136 of the neuraminidase gene. Sequence analysis of virus at passage level E1 did not detect a mixed population and shows only the presence of the codon CTG (translates to glutamine (Q) residue).

to detect the presence of RNA in fore brain, hind brain, lung and liver samples. NAI susceptibility analysis of the virus isolated from the brain samples revealed that unlike the resistant virus from the nasal wash on day 8, the viruses isolated from the brain were fully susceptible to zanamivir and oseltamivir (Table 3), even though the nasal wash, brain samples and internal organs were sampled within hours of each other.

Sequence analysis of the resistant isolate revealed only one amino acid mutation in either the HA or NA genes compared with the stock virus used to infect the animals. The mutation was at residue 136 of the NA and involved a change from Q to L. To ensure that the mutation was present in the original specimen, and not simply an artefact of cell culture passage, sequence analysis was conducted directly on virus from the nasal wash taken at day 8. Examination of the sequence chromatogram for virus from the original specimen demonstrated a mixed viral population, although the majority of the viruses had the mutant codon CTG (translates to L) whilst a minor proportion had the wild type codon CAG (translates to Q) (Fig. 1). Analysis of the sequence chromatogram of the egg grown isolate (following a single passage from the original specimen) revealed no evidence of the wild type virus that was observed as minor population in the nasal wash (Fig. 1). Sequence analysis of the positive RT-PCR product generated following direct amplification of the NA gene from fore brain, hind brain, lung and liver samples revealed only the presence of the wild type strain with no detectable Q136L mutant virus within the population.

4. Discussion

The NA inhibitors are essential drugs for the treatment of A(H5N1) infected patients, although concerns exist that resistance may reduce the effectiveness of antiviral therapy (White et al., 2009). In the absence of human clinical trial data, animal models provide a more realistic alternative to predicting the likelihood of resistant variants being selected under NAI pressure than *in vitro* cell culture experiments. In a previous study we generated NA inhibitor resistance in the same two A(H5N1) viruses, Vn/1203 and Laos/26, following *in vitro* passage of the strains in increasing concentrations of drug (Hurt et al., 2009a). Under zanamivir *in vitro* pressure, two NA mutations (D198G and E119G) were selected and shown to result in reduced zanamivir susceptibility, whilst under oseltamivir *in vitro* pressure, variant viruses with the H274Y NA resistance mutation were selected, and in the case of the Laos/26 virus, the H274Y mutation was paired with another mutation (I222M) which reduced oseltamivir susceptibility even further (Hurt et al., 2009a). Interestingly, none of the residue mutations identified in the previous *in vitro* study were detected in this current *in vivo* study, and similarly the Q136L mutation identified in this study in a zanamivir-treated ferret was not detected in the *in vitro* study.

Mutations at the Q136 residue of the NA have, to date, been rare. Analysis of all N1 sequences on GenBank revealed 5504 strains with the wild type residue Q at position 136, and only 23 of these strains contained a mutation at that residue (11 A(H1N1) strain with Q136K; 11 A(H5N1) strains with Q136H; 1 A(H5N1) strain with Q136R) None of the N1 sequences on GenBank contained a Q136L mutation. The role of the Q136 residue in zanamivir resistance had not been recognized until recently when a number of seasonal A(H1N1) isolates with a Q136K mutation were detected and found to have significantly raised zanamivir IC_{50} values (Hurt et al., 2009c; Okomo-Adhiambo et al., 2010). The Q136K mutant viruses did not come from treated patients and therefore were not selected under zanamivir pressure, unlike the Q136L mutant detected in this study. Unusually, although the Q136K mutant was detected in the isolates, it could not be detected in the clinical specimens suggesting that either it was generated during cell culture or that it was present only at low levels in the specimens (Hurt et al., 2009c; Okomo-Adhiambo et al., 2010). In contrast the mutant Q136L virus in this current study was clearly evident in the original specimen, even though it was part of a mixed population. Culture of the original sample in eggs appeared to select for the Q136L virus, similar to the selective advantage of the Q136K mutant in cell culture (Hurt et al., 2009c). It would be of interest to assess the *in vivo* viral fitness of the Q136L mutant strain in comparison to the wild type, particularly given that the Q136K mutant demonstrated similar infectivity and transmissibility in ferrets to the wild type (Hurt et al., 2009c). While the levels of zanamivir resistance were similar for both the Q136L and Q136K mutants, one difference was that the Q136L in this current study caused a minor increase in oseltamivir IC_{50} (16-fold), whereas the Q136K mutation had no effect on oseltamivir susceptibility (Hurt et al., 2009c). The Q136K mutation was thought to cause mobility of the R156 and D151 residues which disturbed the interactions between these residues and zanamivir and lead to the observed increases in IC_{50}. Because the Q136K virus remained susceptible to oseltamivir it was thought the interactions between the D151 side chain and oseltamivir were maintained, although it is possible that the Q136L mutation may disturb these interactions and cause the increase in oseltamivir IC_{50} value.

Unlike oseltamivir, where approximately 80% of the oral prodrug is readily absorbed and therefore available systemically (He et al., 1999), an inhaled dose of zanamivir is deposited primarily in the oropharynx (78% of dose), with 13% of the dose reaching the

bronchi or lungs (Cass et al., 1999a), and only 4–17% being systemically absorbed (Cass et al., 1999b). The localised mode of action of inhaled zanamivir appears suitable for normal human seasonal influenza strains, but may be insufficient in inhibiting A(H5N1) viruses that have the capacity to undergo systemic spread (de Jong et al., 2006). The lower levels of zanamivir in the non-respiratory organs may provide an environment suited to resistance selection should a strain, such as A(H5N1), replicate in this region of the body. An interesting observation in this study was the localised detection of the resistant Q136L mutant virus in the nasal wash, whereas only susceptible wild type virus was detected in the fore and hind brain, liver or lung on the same day. This suggests that resistant virus probably arose in the respiratory tract where the virus was under greatest zanamivir selective pressure, and that the variant was either not selected for in other parts of the body, or alternatively that there had been insufficient time for the mutant to spread to the other regions of the body. Formulations of zanamivir that involve intravenous administration have been shown to achieve a more systemic distribution (Cass et al., 1999b), and have been effective in treating A(H5N1) infections in macaques (Stittelaar et al., 2008).

Oseltamivir resistance was not detected in any of the ferrets under treatment, a result similar to that obtained by Govorkova et al. (2007) who also found that emergence of resistance in ferrets under oseltamivir treatment was not detectable at the virus population level, although in that study clonal analysis did reveal some resistant strains at very low proportions within the viral population ($\leq$10%). In human studies, oseltamivir resistance has been detected at a significantly higher frequency than zanamivir resistance (Hurt et al., 2006; McKimm-Breschkin, 2000; Sheu et al., 2008). This difference may be due to the significantly greater use of oseltamivir compared to zanamivir in the community (Hurt et al., 2009b). However as a result of the increased frequency of oseltamivir resistance in circulating strains, the use of zanamivir is likely to increase in the future, and as such it remains important to monitor viruses for zanamivir resistance. Given the detection of the Q136L mutant in this study and the Q136K mutants reported recently in seasonal A(H1N1) strains (Hurt et al., 2009c) it would appear that the Q136 residue may be one of the key residues to monitor in both A(H5N1) and seasonal and pandemic A(H1N1) viruses.

Further work is necessary to better understand and determine the optimal treatment for A(H5N1) infected patients, particularly in light of previous animal studies that have demonstrated the benefits of modified dosing regimes compared to those developed for the treatment or prophylaxis of seasonal influenza (Boltz et al., 2008; Govorkova et al., 2007). Only the highest oseltamivir dose in this study was found to have a significant impact on ferret survival following Vn/1203 challenge, and neither oseltamivir nor zanamivir (at any dose) appeared to impact significantly on the duration of viral shedding in the less pathogenic Laos/26 virus. This study also demonstrates that A(H5N1) strains can develop novel NAI resistance mutations that have not been previously identified in seasonal influenza viruses, further reinforcing the need for future *in vivo* and *in vitro* NAI resistance studies on strains of a novel subtype.

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