Naturally Occurring Antiviral Drug Resistance in Avian H5N1 Virus

Jane M Rayner, Jinxia Zhang, Gavin JD Smith, Wai-Lan Wu, Siu-Ying Lau, Pengxi Zhao, Malik JS Peiris, Yi Guan, Honglin Chen

State Key Laboratory for Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong, Hong Kong SAR, China

Resistance to the neuraminidase inhibitor, oseltamivir, was found in H5N1 virus isolated from infected patients in Vietnam but no recent avian H5N1 isolates have been reported to possess known neuraminidase inhibitor resistance mutations. It is still not clear whether the NA1 gene neuraminidase mutation, His274Tyr, detected in H5N1 infected human cases originated during disease treatment or came from the avian virus source. A 2002 HK chicken H5N1 isolate was found to contain the His274Tyr mutation on NA1 and resistance to oseltamivir was confirmed using a cell-based assay. To investigate if His274Tyr might naturally occur at low levels mixed with wild type in H5N1 poultry infections we examined isolates from different hosts and geographical locations using a differential RT-PCR assay. The His274Ty quasi species was more frequently recognized in isolates from infected chickens than in isolates from ducks and geese, but no geographical difference was observed. Close surveillance of mutants in the virus population, combined with pursuit of alternative therapies, is essential to H5N1 pandemic containment strategies.

Introduction

Outbreaks caused by avian influenza H5N1 virus have been reported in poultry in nine southeast Asian countries since 2003 [1,2,3]. Increasing evidence shows H5N1 virus is endemic in poultry in this region; it may not be eradicable from avian hosts by regular control measures. The outbreak in migratory birds at Qinghai Lake in 2005 led to further expansion of the geographical distribution of H5N1 virus from Asia to Europe, the Middle-east and Africa [4,5]. Meanwhile, over 300 human infections by H5N1 virus have occurred in affected countries since 2003 [6]. There is concern that the H5N1 virus, or a derivative of it, may stand to be the next pandemic influenza virus [7]. It may still be some time before an effective vaccine, in adequate quantities, is available. This leaves protection in the face of a pandemic largely at the therapeutic level through antiviral treatment, placing emphasis on stockpiling antiviral drugs, particularly those for which resistance is not regularly detected [8]. Oseltamivir, a neuraminidase inhibitor, blocks exit of maturing virus particles from infected cells. In vivo and in vitro studies on H5N1 isolates from Vietnam and index strains from Hong Kong (1997 and 2003) have shown that oseltamivir effectively inhibits virus replication [3, 9-11]. However, resistance to oseltamivir has been observed, albeit rarely, during treatment of H3N2 and H5N1 influenza virus infections [12, 13-14]. This study examines H5N1 sequence data to investigate the occurrence and distribution of oseltamivir resistant mutants isolated in Asia since 1997. We found that oseltamivir resistant mutant strains do occur and their prevalence varies with host-species. This information is relevant to influenza pandemic preparedness, particularly with regard to choice of suitable pharmaceutical agents for stockpiling, and emphasizes the need for alternative therapies, including novel drugs and an effective vaccine.

Materials and Methods

Sequence analysis. Sequences of H5N1 viruses sampled from 1997 onwards were selected from our collection and public databases. Sequence data were aligned with and residue analysis performed using BioEdit (Version 7) [15]. Residues Glu 119, Arg 292, His 274 and Arg 152 of the NA were selected to screen for predicted oseltamivir resistant mutants.

Detection of H274Y mutant with specific PCR. The His274Tyr mutation on subtype 1 neuraminidase (N1) is caused by a C to T substitution at nucleotide position 763. Differential PCR was performed with two forward primers, primer C (5' GAATTGGATGCTCCTAATTATC 3') and primer T (5' GAATTGGATGCTCCTAATTATT 3') that differ by one nucleotide at the 3' end, and a single reverse primer (5' AGAGGACACCGGACCACAACTAC 3'). The primer pairs were tested with His 274 and Tyr 274 reference templates to optimize conditions for primer specificity in mixtures of the two templates (95°C 10 min, 1 cycle; 95°C 1 min, 62°C 1 min, 72°C 1 min, 35 cycles; 72°C 10 min, 1 cycle; Fig. 1). RNA extraction and cDNA synthesis from virus samples were performed as described previously [2].

Inhibition of H5N1 virus infection in MDCK cell culture. Virus stocks were propagated in embryonated chicken eggs and $TCID_{50}$ in MDCK cells was determined. Oseltamivir carboxylate (Roche) was tested at concentrations of 0.1 nM to 100 uM in a cell-based virus reduction assay, modified from that of Yen and co-workers [11]. Briefly, triplicate MDCK monolayer cultures in 96-well format were infected with 100 $TCID_{50}$ virus doses (1 hour, 37°C, 5% CO_2), then the inoculum removed and cell layers washed with culture media (MEM, Gibco BRL). Two hundred microlitres of culture media containing the appropriate dilution of oseltamivir was added to wells, and cultures incubated for 3 days (37°C, 5% CO_2). The HA titers of individual well supernates were tested in duplicate.

Quantification of wild type (C type) and mutant (T type) NA gene by quantitative PCR. Quantitative PCR (qPCR) reactions were performed with a Roche Lightcycler system (Roche) using a Lightcycler Faststart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. For better discrimination between the wild type and mutant NA genes, Locked Nucleic Acid (LNA) primers (Proligo) were used. Wild type and mutant NA genes were cloned into the TOPO PCR 2.1 vector (Invitrogen), sequenced and used as standards for quantification. Mutant and wild type NA gene copy number were calculated with Lightcycler software using the wild type and mutant plasmids as a standard. PCR specificity was checked by melting curve analysis and gel electrophoresis.

Results

Detection of an oseltamivir resistant H5N1 isolate. To date, four neuraminidase residue changes associated with resistance to oseltamivir have been characterized; Glu119Val, Arg292Lys, His274Tyr and Arg152Lys. These appear to be NA subtype specific; Arg292Lys and Glu119Val to N2 and His274Tyr to N1 [16-18]. Published sequences of avian H5N1 virus isolates do not indicate the presence of resistance-associated mutations and the isolates tested are sensitive to oseltamivir inhibition both in vitro and in vivo [10,11]. However, the isolation of oseltamivir-resistant H5N1 viruses from humans in Vietnam indicates that oseltamivir resistance could be an emerging problem [13,14]. As oseltamivir is so important to pandemic preparedness, we wished to investigate if resistant mutant H5N1 viruses might be present in other species and areas in Asia, with a view to determining the potential of a naturally-resistant pandemic strain arising in the future. Available sequences of H5N1 viruses isolated since 1997 were examined. One isolate, A/chicken/Hong Kong/3123.1/02, was found to bear the His274Tyr mutation which conveys resistance to neuraminidase inhibitor drugs in N1 subtype viruses [16-18]. Drug sensitivity tests revealed that this isolate possessed a high degree of resistance to oseltamivir inhibition, with an EC50 of 124.9uM. In comparison, the A/chicken/Vietnam/37/04 isolate, containing the wild type residue at position 274, has an EC50 of 0.4uM oseltamivir.

Figure 1. Sequencing traces of the neuraminidase gene showed a mixture of wild type (C) and H274 Y (T) mutant in an H5N1 ISOLATE (A). A C T Mutation at this position confers resistance to oseltamivir in H5N1 subtype viruses. Primers for wild type (C) and mutant (T) were designed with one nucleotide difference at the 3' end of each primers were used with a common reverse primer derived from a non-variable region. Conditions allowing specific detection of wild type or mutant were potimized with templates containing only mutant or wild type and mixtures of these templates (B). Detection of wild type (primer W) AND his274tYR TYPE (PRIMER m) in H5N1 isolates and one orignial swab by PCR (C). 1: A/chicken/Vietnam/37/04, 2: A/chicken/Hong Kong/518.1/02, 3: A/chicken/485.2/02, 4: A/chicken/Vietnam/272/05, 5: A/chicken/Hong Kong/WF75/02, 6: A/chicken/Hong Kong/WF75/02 (original swab).
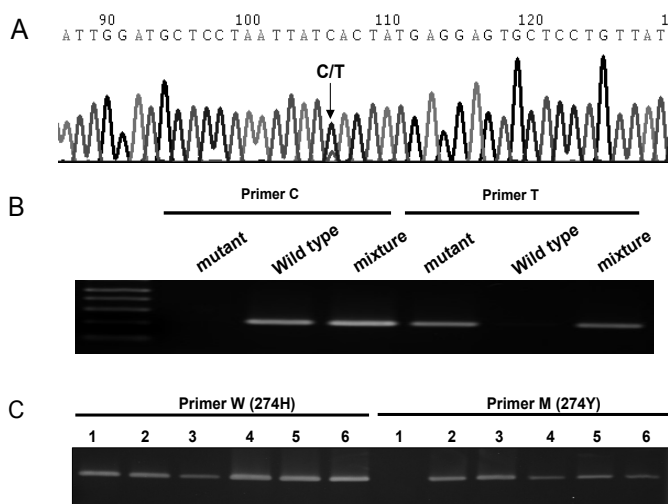
Presence of His 274Tyr mutant mixed with wild type virus in H5N1 isolates. The human H5N1 virus isolated from patients treated with oseltamivir has been identified as consisting of mixed populations of His 274 wild type and Tyr 274 oseltamivir-resistant virus [13,14]. As direct PCR product sequencing is the most regularly used method of determining influenza virus sequence, and sequence traces are rarely consulted, only the dominant sub-population at each nucleotide is represented in analyzed sequences, potentially obscuring the presence of low-frequency variant populations (Figure 1A). A protocol was developed and verified for rapid screening of H5N1 isolates which may contain both mutant Tyr 274 and wild type His 274 NA1 (Figure 1B). Where available, original cloacal swabs corresponding to isolates identified as containing the His274Tyr mutant were compared with viral stocks to confirm that His274Tyr mutant virus was present in the original samples prior to viral propagation in embryonated eggs (Figure 1C).

Overall, 54.3% of tested isolates contained detectable levels of His274Tyr variant virus, but the distribution was skewed, with variant detected most frequently in chicken isolates, less often in human and more rarely in duck and goose isolates (Table 1). His274Tyr mutant virus was not detected in a sample of nine H1N1 isolates tested using a similar procedure (data not shown). Species-specific analysis revealed that variant His274Tyr virus was not detected in chickens in the 1997-1999 period (data not shown,). Following emergence of the mutant in 2000, prevalence in chickens then rose rapidly, with just under 10% of 2000-2001 viruses testing positive by PCR screening, and variant frequency plateauing at a high of 74% over the 2002-2005 period. Geographical distribution of His274Tyr mutant virus was also analysed, but no distinct pattern could be discerned (data not shown).

Table 1. Specific PCR screening for presence of His274Tyr mutation in H5N1 viruses isolated from various hosts.

Species	Total isolates	Positive	% positive
Human	19	8	42.1
Chicken	195	125	64.1
Duck/Goose	97	36	37.1
Total	311	169	54.3

Quantitative PCR was applied to quantify the relative abundance of His274Tyr mutant and the wild type in virus stocks. In essentially all isolates containing mixed species of wild type and mutant, the mutant represented less than 1% of the virus population, and in many cases less than 0.1% (data not shown).

Discussion

Preparedness for a potential H5N1 influenza pandemic caused by currently-circulating H5N1 viruses has been recommended by the WHO. One well-publicized focus is the stockpiling of antiviral drugs, particularly inhibitors of viral neuraminidase. One

such neuraminidase-inhibitor, oseltamivir, has been considered as one of the few options available for the containment of human-to-human transmission. This study demonstrates that oseltamivir resistant strains bearing the His274Tyr mutation are present in some H5N1 virus populations, albeit at low levels, in the absence of evidence of exposure to the drug. Oseltamivir is not known to have been introduced into the Hong Kong market prior to 2002, but we have detected His274Tyr mutants in virus populations isolated as early as 2000, which suggests that this mutant occurs naturally. Previous studies have shown that oseltamivir-resistant mutants have a growth and virulence disadvantage [19-22]. Given the continuous circulation of H5N1 viruses in poultry and wild birds in the southeast Asia region, there is a chance that His274Tyr mutants could gain the ability to increase in abundance if they obtain other adaptive changes. Even if variant viruses do not become dominant in mixed populations, they may still hamper clinical treatment with oseltamivir by increasing the overall resistance of the population. Isolation of one resistant mutant strain, A/chicken/Hong Kong/3123.1/02, from an infected chicken suggests the possibility that such mutants may possess the potential to become the main population, at least in chickens. Major H5N1 outbreaks in poultry in 1997 and 2001-2002 in Hong Kong were controlled in a timely manner and this might have stopped further expansion of such mutants [23,24]. Antiviral drugs, if appropriately applied and tightly controlled, remain important components of pandemic preparedness. However, the presence of drug-resistant mutants in the pool of potential pandemic H5N1 strains provides additional "options" for the predicted pandemic strain, especially with regard to oseltamivir-resistance. In anticipation of this possible scenario, further control options must be implemented, including stockpiling a wider range of drugs (zanamivir in particular), development of alternative pharmaceuticals, emphasis on vaccine development and, most importantly, increased surveillance for the presence of His274Tyr mutants mixed with wild type strains among new H5N1 isolates.

Acknowledgements

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