Original article

A comprehensive surveillance of adamantane resistance among human influenza A virus isolated from mainland China between 1956 and 2009

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Background: Adamantane-derived drugs have been used for treatment and prophylaxis of influenza A virus infection for many years worldwide. Rapid surveillance of antiviral drug resistance is important for appropriate clinical guideline development. Here, we retrospectively assessed adamantane resistance among different influenza A subtypes (H1N1, H3N2 and H5N1) over 53 years (1956–2009) in mainland China.

Methods: A total of 1,451 viruses, including 773 H3N2 viruses, 647 H1N1 viruses and 31 human H5N1 viruses, were analysed by matrix gene sequencing and assayed for drug resistance.

Results: Our results show that the prevalence of adamantane-resistant H3N2 viruses was low between 1956 and 2002, but substantially increased in 2003 to the extent that since 2006 all H3N2 viruses have been drug resistant. The percentage of adamantane-resistant H1N1 viruses also increased from 50.0% in 2004 to 98.7% in 2007; however, this decreased to 46.7% in 2009. Only three adamantane-resistant H5N1 viruses have been detected since 2003, when the first case of human H5N1 virus infection was detected in mainland China. Phylogenetic analysis demonstrated that the increase of adamantane-resistant isolates was caused by point mutations or intrasubtype reassortment instead of intersubtype reassortment.

Conclusions: Because of the high percentage of adamantane-resistant H3N2 and H1N1 viruses in mainland China, the use of amantadine and rimantadine drugs for prophylaxis and treatment of current seasonal influenza A infection is not recommended.

Introduction

Influenza A virus, a common pathogen in humans and other species [1], causes significant morbidity and mortality in humans worldwide [2]. Although annual vaccination is the primary strategy for preventing influenza infections, antiviral drug therapy has also been shown to be effective in influenza prevention and treatment. Currently, two classes of antiviral drugs are in clinical use, adamantanes (that is, amantadine and rimantadine) and neuraminidase inhibitors (that is, oseltamivir and zanamivir). Amantadine and rimantadine have been successfully used worldwide for treatment and prophylaxis of influenza A virus infection for >30 years [3,4], and have been stockpiled for use in future pandemics in some countries [5]. These drugs function by blocking the M2 protein, which is the proton channel of the influenza A virion, inhibiting the pH change necessary for the uncoating process and blocking virus replication by preventing release of viral RNA into the cytoplasm of infected cells [6]. These drugs are inexpensive and chemically stable; however, the emergence of resistance and adverse effects create concern regarding their use in the clinic [7]. The molecular mechanism of viral resistance to these drugs has been well-characterized and is associated with one or multiple amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane domain of the M2 protein [8,9].

In countries and areas with active surveillance, the rate of resistance has been shown to have increased substantially in recent years, particularly in H3N2 viruses [1,10,11]. Rates of resistance among influenza A H1N1 viruses are lower than with H3N2 [1,12]. In addition, during 2003–2009, 38 cases of human infection with avian influenza H5N1 virus were confirmed in China [13–16], and viruses of this subtype that are resistant to adamantanes appeared to spread rapidly in Southeast Asia, China and neighbouring countries [17,18].

Since the 1950s, influenza surveillance has been routinely conducted by the Chinese National Influenza Center (CNIC; Beijing, China), which is a member of the Global Influenza Surveillance Network. A large number of influenza viruses isolated from mainland China are stored in the CNIC. In order to summarize the prevalence of circulating viruses resistant to adamantane-derived drugs and to provide scientific evidence for the role of drug administration, we screened for adamantane resistance among influenza A H1N1, H3N2 and H5N1 viruses collected nationwide between 1956 and 2009. It is the first time that the CNIC has summarized and published comprehensive adamantane resistance data of three subtypes of influenza A virus isolated from mainland China over 50 years.

Methods

National Influenza Surveillance Network in mainland China

Influenza is categorized as one of the Class C notifiable infectious diseases in mainland China. Influenza surveillance began in 1952 and the CNIC was established in 1957 because of the 'Asian pandemic'. In 2000, a National Influenza Surveillance Network was established, which has gradually expanded to the extent that it currently consists of 411 laboratories and 556 sentinel hospitals. Each sentinel hospital reports influenza-like illness data and collects weekly specimens. Network laboratories isolate virus from these specimens and conduct virus characterization by using haemagglutination inhibition assays. All the data is reported to CNIC through an internet-based National Influenza Surveillance information system and all virus isolates are submitted for further antigenic and genetic analyses.

Viruses

Viruses isolated from mainland China between 1956 and 2009 were selected from our National Influenza Surveillance database; if there were >10 isolates of a given subtype in 1 year in one province, we randomly selected 10 isolates from this subtype. A total of 1,451 influenza A isolates (H3N2 n=773, H1N1 n=647 and H5N1 n=31) were propagated in either embryonated eggs or Madin–Darby canine kidney cells and were antigenically subtyped using haemagglutination inhibition assays [19].

Viral RNA extraction

Viral RNA was extracted from 100 µl of fresh virus culture medium using an RNeasy Mini kit (Qiagen, Hilden, Germany) and then washed in 35–50 µl RNase-free ultra pure water, according to the manufacturer's protocol.

M2 gene reverse transcriptase PCR

Reverse transcriptase (RT)-PCR was performed as described previously [20]. Briefly, a 1,018 nucleotide amplicon of the matrix (M) gene covering the region encoding the transmembrane domain of M2 was targeted for RT-PCR amplification. Primer sets (UF12: 5′AGCAAAAGCAGG-3′, MF: 5′-CAGGTAGATATTGAAAGAT-3′ and MR: 5′-GTAGAAACAAGGTAGTTTT-3′) were synthesized by TAKARA Bio Inc. (Kyoto, Japan) to amplify the M2 gene. Reverse transcription was carried out by UF12 for a 60 min incubation at 42°C. M-gene-specific primers MF and MR were used for target product amplification, the reaction procedure was as follows: 94°C for 5 min; 94°C for 30 s, 50°C for 30 s, 72°C for 50 s, 35 cycles; 72°C for 7 min and stop at 4°C.

PCR product purification and sequencing

PCR products were purified with agarose gel according to the manufacturer's protocol (QIAquick® Spin Handbook; Qiagen). The purified PCR product was sequenced in both directions using Sanger's dideoxy-mediated chain termination method on an automatic sequencing machine (ABI 3730XL; Applied Biosystems, Carlsbad, CA, USA).

Sequence analyses

We sequenced the M genes of 1,451 viruses, and then analysed the M2 ion channel amino acid, which is associated with adamantane resistance. A phylogenetic tree of 1,339 viruses was constructed to analyse the evolution relationship among adamantane-resistant viruses of different subtypes with the length of M gene nucleotide sequence ≥860 base pairs. Nucleotide and amino acid sequences were analysed using DNASTAR Lasergene 7.1 (DNASTAR Inc., Madison, WI, USA) and Phylip3.68 (Department of Genome Sciences, University of Washington, WA, USA).

Drug resistance assays

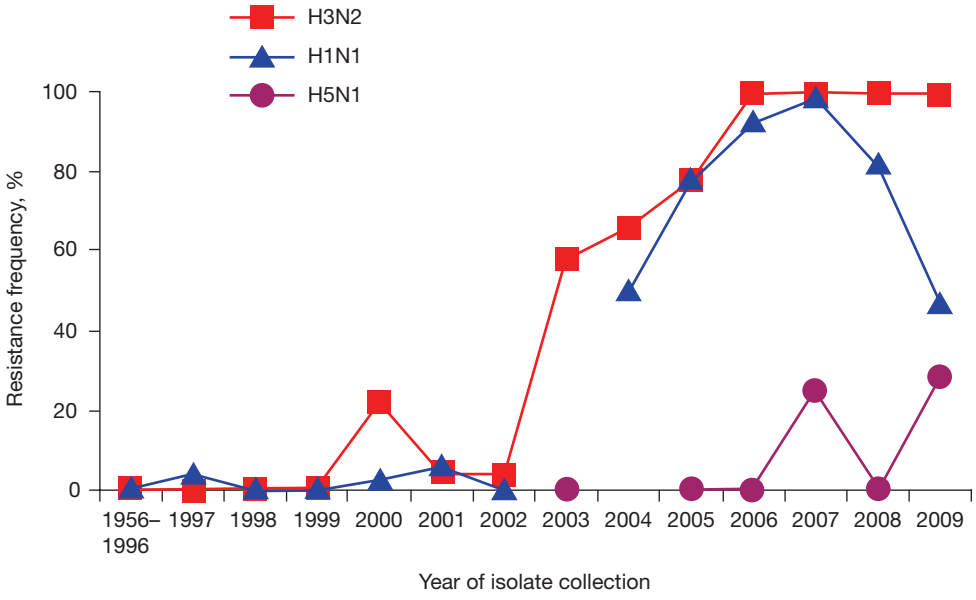
A biological assay, previously described [10], was used to confirm antiviral resistance results and rimantadine (Sigma, Munich, Germany) drug susceptibility

Table 1. Frequency of adamantane-resistant viruses of different influenza A subtypes and year of isolation in mainland China between 1956 and 2009

Influenza A subtype	Frequency per year of isolation													
	1956–1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
H3N2	0/36[a]	0/11	0/24	0/10	2/9 (22.2)	1/23 (4.3)	2/50 (4.0)	40/69 (57.9)	31/47 (65.9)	77/99 (77.8)	100/100 (100.0)	191/191 (100.0)	52/52 (100.0)	52/52 (100.0)
H1N1	0/69	1/25 (4.0)	0/17	0/2	1/41 (2.4)	2/35 (5.7)	0/17	NS	1/2 (50.0)	24/31 (77.4)	96/104 (92.3)	74/75 (98.7)	112/137 (81.8)	43/92 (46.7)
H5N1	NS	NS	NS	NS	NS	NS	NS	0/1	NS	0/5	0/11	1/4 (25.0)	0/3	2/7 (28.6)

Values presented as *n*/total *n* (%) or *n*/total *n*. [a]Between 1968 and 1996. NS, none sequenced.

Figure 1. Frequency of adamantane-resistant viruses of different influenza A subtypes and year of isolation in mainland China between 1956 and 2009



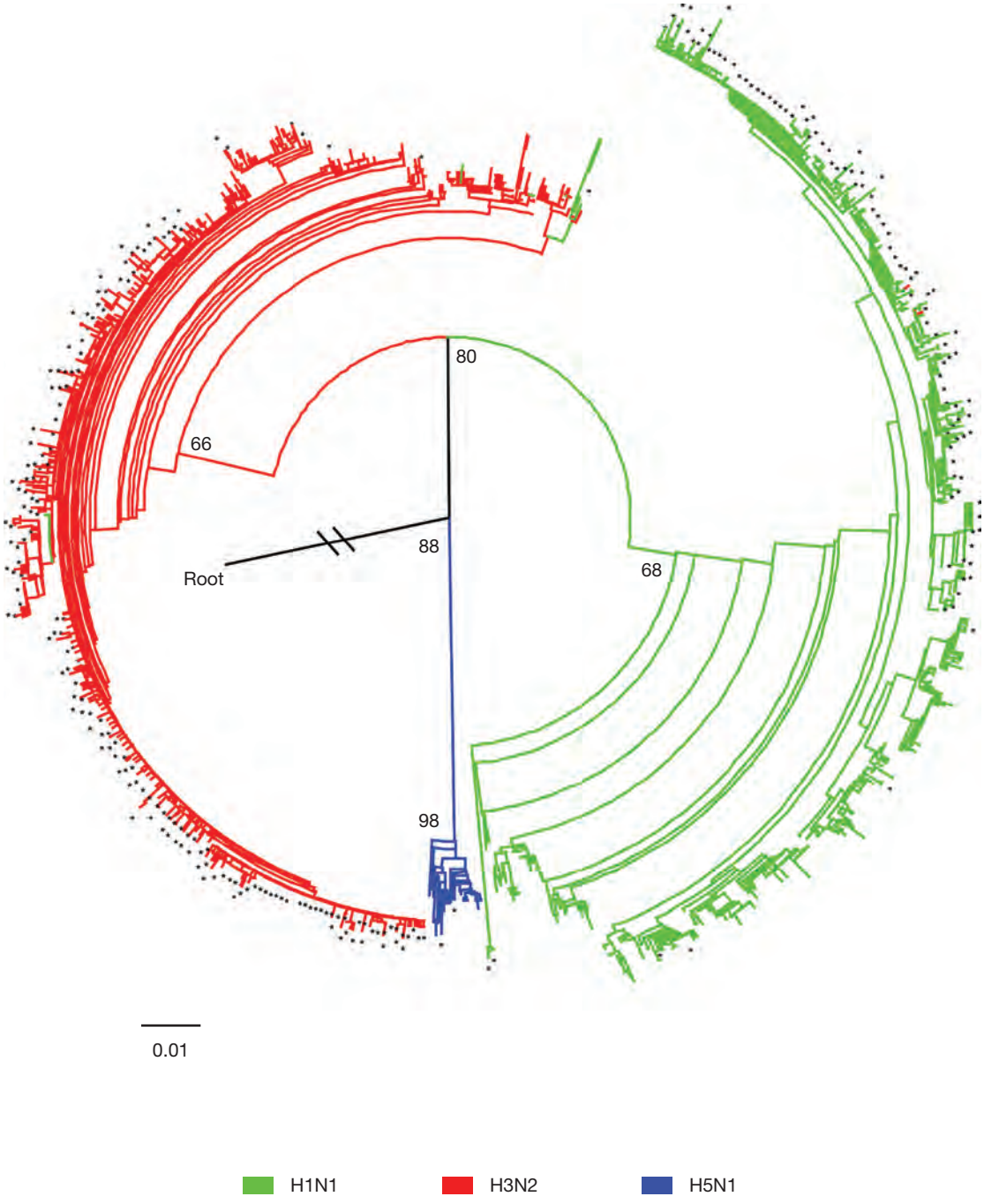
on 20 virus isolates randomly selected from sequenced H3N2 subtype virus isolates. Briefly, Madin–Darby canine kidney cells were cultured in 24-well plates until 80–90% confluence and 100 µl of the maintenance medium containing 0, 0.2, 2.0 or 20 µg/ml rimantadine were added to each well. Influenza viruses diluted to 1:2, 1:20 or 1:200 in medium containing 2 µg/ml tosyl phenylalanyl chloromethyl ketone trypsin were then added to each well and incubated for 1 h at 37°C in 5% CO_2. Subsequently, an additional 300 µl of normal cell maintaining medium was added and plates were incubated for a further 36 h. The virus sensitivity to rimantadine was assessed using haemagglutination titre assays as described previously [10].

Results

Nucleotide and amino acid sequence analyses

Table 1 and Figure 1 show the percentages of adamantane-resistant virus from different subtypes identified by gene sequence analysis. Adamantane-resistant viruses were not observed in all these subtypes before 1997; however, the percentage of adamantane-resistant H3N2 virus notably increased to 57.9% in 2003. It then increased over the following 3 years and has remained at 100% since 2006. Similar to H3N2, the percentage of adamantane-resistant H1N1 virus substantially increased from 50.0% in 2004 to 98.7% in 2007; however, it decreased to 46.7% in

Figure 2. Phylogenetic relationship of M genes of 1,339 influenza A viruses isolated in mainland China



Analysis was based on nucleotides 79–937 (860 base pairs) of the matrix (M) gene. The tree was rooted to B/Hong Kong/5/1972. The numbers above the branch nodes indicate neighbour-joining bootstrap values. Scale bar, 0.01 substitutions per site. Asterisks indicate adamantane-resistant viruses.

2009. Sporadic adamantane-resistant H5N1 viruses were identified in 2007 and 2009.

Overall, 891 viruses contained a single amino acid substitution that correlated with drug resistance: L26F, 1; V27A, 6; A30T, 1; and S31N, 883. A total of 14 viruses contained two amino acid substitutions at positions S31N and V27A; two of these were sampled from different hospitals in 2007 and 12 were from the same hospital at different time points during 2008. No viruses containing more than two amino acid substitutions were observed. Amino acid substitution at position S31N was frequently observed in the resistant viruses (n=897, 99.1%), which is consistent with a previous study [1].

Phylogenetic analyses

To explain the evolutionary relationship among different subtypes, a phylogenetic tree of the M gene containing 1,339 sequences of different subtypes was generated by Phylip3.68 neighbour-joining (Department of Genome Sciences, University of Washington; Figure 2) [21]. Our phylogenetic analysis of the M segment clearly showed that H3N2, H1N1 and H5N1 formed three independent clades. Interestingly, 17 H1N1 viruses were observed in the H3N2 clade and three H3N2 viruses were found in the H1N1 clade. This phenomenon indicated that intersubtype reassortment might have occurred between H1N1 and H3N2 viruses and the new reassorted virus might have obtained the M gene from either the H1N1 or H3N2 virus; however, H5N1 virus did not gain M genes from H3N2 and H1N1 viruses by intersubtype reassortment or *vice versa*.

Details of the intersubtype reassortment viruses can be found in Additional file 1. Intersubtype reassortment events have occurred since 1974 and there were five such events in 2007. The majority of intersubtype reassortment events occurred in different years and places.

Biological drug resistance analyses

Biological resistance assays were performed using 20 H3N2 strains. The results showed that the genotypes and phenotypes of adamantane resistance were 100% consistent (partial results are shown in Additional file 1).

Discussion

Although intrasubtype reassortment is common in human influenza A virus [22–24], phylogenetically, the M gene for H3N2, H1N1 and H5N1 was found in only a few strains that seemed to be generated by intersubtype reassortment between H1N1 and H3N2, as shown in this and previous studies [25]. Since 1974, there have been 20 intersubtype reassortment events between H3N2 and H1N1, occurring in different years and places; however, these viruses were not maintained in human populations. It has been suggested that the H3N2 virus acquires the sensitive M gene from H1N1, and such a virus might not replicate and/or transmit effectively. This might be one reason that can explain why, for H3N2, the percentage of adamantane-resistant virus was maintained at 100% for 4 years since 2006 in mainland China.

The increasing rate of drug-resistant H3N2 viruses is much greater than that of H1N1 viruses. One possible explanation is that the H3N2 viruses were predominant in most seasons between 2003 and 2008 in China, especially 2003, 2004, 2005 and 2007 (YS, unpublished observations). For example, in 2003 alone, influenza A accounted for 84.3% of the influenza viruses isolated from mainland China and 96.7% of those were H3N2 viruses (YS, unpublished observations).

The molecular mechanism of viral resistance to adamantane-derived drugs has been well-characterized and is associated with one or multiple amino acid substitutions at positions 26, 27, 30, 31 or 34 in the transmembrane domain of the M2 protein. It is still unclear why a single amino acid substitution at position S31N is frequently observed in the resistant viruses. In this study, there were 14 cases where the virus had acquired two amino acid substitutions, at positions S31N and V27A, but these viruses were not detected and did not become dominant.

In mainland China, a similar pattern in H1N1 adamantane-resistant virus epidemics has been observed to that reported in Australia, Europe and the US, but with a 1-year delay [26]. The clade 2C A/Hong Kong/2652/2006-like viruses predominated in 2008 in China; within this clade, 85.3% were adamantane-resistant viruses. Only 10% of the clade 2B A/Brisbane/59/2007-like viruses were found to be adamantane-resistant. In 2009, A/Brisbane/59/2007-like viruses became the predominant influenza strain in China and, consequently, the overall percentage of adamantane-resistant H1N1 viruses decreased to 46.7%.

Our findings suggest that >70% of the H5N1 viruses isolated from China are sensitive to adamantane-derived drugs. This might be attributable to specific characteristics, such as its highly sporadic nature, avian origin and lack of efficient transmission among humans. These factors result in a short exposure history of the virus to adamantane-derived drugs. In addition, most sporadic human H5N1 cases are treated with Tamiflu® rather than adamantane-derived drugs in mainland China.

Recently, it was found that >99.7% of circulating pandemic H1N1 viruses (2009) globally, were resistant to adamantane [27–29] and similar data was observed in mainland China alone. In general, our data also shows that a high percentage of seasonal influenza

isolates are resistant to adamantane-derived drugs; hence, the use of adamantane-derived drugs is not recommended for antiviral therapy for seasonal influenza virus. In addition, our surveillance data shows that seasonal influenza H3N2 and influenza B are still sensitive to neuraminidase inhibitors (YS, unpublished observations). Because of the limited choice of antiviral therapy in treatment of influenza A, an enhanced surveillance of drug resistance in virus isolates is necessary to identify the best treatment options and allow for the development of new classes of antiviral drugs.

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Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: A list of intersubtype reassortment viruses and rimantadine-resistance assay results can be found at http://www.intmedpress.com/uploads/documents/AVT-10-OA-1527_Lan_Add_file1.pdf

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