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Amantadine-resistance among H5N1 avian influenza viruses isolated in Northern China☆

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Abstract

We tested the amantadine-resistance among avian influenza A (H5N1) viruses isolated from chicken in Hebei Province of Northern China from 2001 to 2005, and investigated the amantadine use in this area. Plague reduction assay in MDCK cells showed that 83.3% isolates (5/6) were amantadine-resistant strains. The M2 sequence analysis revealed that four of five resistant isolates contained the point mutations (Ser to Asn) at position 31 that could confer resistance to amantadine. These results indicated that the incidence of amantadine-resistant viruses isolated in Northern China was particularly high. In the investigation of amantadine use, we found that amantadine was used extensively in poultry farms in this area, which maybe was one of reasons of the high amantadine-resistance incidence.

Keywords: Amantadine-resistance; H5N1 avian influenza viruses; Northern China; M2 gene

1. Introduction

Avian influenza A virus, particularly highly pathogenic H5N1 subtype, has emerged as an important pathogen in poultry industry and constitutes a major pandemic threat to humans. Extensive surveillance and genetic studies revealed that H5N1 viruses had become endemic in poultry in Southern China and Southeast Asia since 2003 (Li et al., 2004; Smith et al., 2006; Guan et al., 2002). Justifiably, the long-term endemicity of H5N1 virus increases the opportunity for the emergence of potential pandemic strains through further genetic mutation or reassortment. In a pandemic situation, when vaccines against the new strain are not yet available or as long as the available vaccine is in short supply, antiviral drug may play an important role in the early phase (Hurt et al., 2007).

Currently, two classes of antiviral drugs are available to treat influenza A infection: the inhibitors of M2, amantadine and rimantadine, and the neuraminidase inhibitors, zanamivir and oseltamivir (Monto, 2003). In vitro studies showed that amantadine had inhibitory effect on H5N1 influenza virus in cell culture (Subbarao et al., 1998; Ilyushina et al., 2006). For human H5N1 virus infection, there were few clinical evaluations about the therapeutic value of amantadine, and so we cannot exclude the possibility of amantadine use in a H5N1 pandemic situation. Therefore, it is necessary to reevaluate the role of amantadine in H5N1 virus infection and analyze the geographic distribution of amantadine-resistant variants (Cheung et al., 2006).

Recent studies reported that some H5N1 viruses from human and poultry in Southern China and Southeast Asia were resistant to amantadine, and we also could obtain some genetic evidence for resistance to amantadine from GenBank. However, information from Northern China was particularly less. So it is critical to investigate the frequency of amantadine-resistant isolates in Northern China, because it could answer the key question regarding the drug-resistant variants in and out of Southern China, the hypothetical influenza epicenter. In this study, we tested the incidence of amantadine-resistance among H5N1 avian influenza A viruses isolated in Hebei Province of Northern China, and investigated the amantadine use in the area. We also tried to clarify the possible reasons of high amantadine-resistance incidence.

2. Materials and methods

2.1. Drug

Amantadine hydrochloride was purchased from Alfa Aesar (A Johnson Matthey Company, USA, Lot: 10118375; Biotech

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Table 1
Amantadine sensitivity assay of H5N1 viruses by plague reduction assay in MDCK cells

H5N1 viruses	M2 mutation that confers resistance to amantadine	IC_{50} (mean ± S.D., μM)[a]	Antiviral sensitivity	Date of virus isolation
A/Chicken/Hebei/718/2001	–[b]	0.044 ± 0.0058	Sensitive	July 2001
A/Chicken/Hebei/108/2002	S31N	59.7 ± 5.1	Resistant	January 2002
A/Chicken/Hebei/326/2005	S31N	20.3 ± 2.9	Resistant	March 2005
A/Chicken/Hebei/706/2005	–	28.0 ± 0.32	Resistant	July 2005
A/chicken/Hebei/102/2005	S31N	41.0 ± 2.0	Resistant	October 2005
A/Chicken/Hebei/126/2005	S31N	71.6 ± 3.5	Resistant	December 2005

[a] IC_{50} values were determined by plaque reduction assay in MDCK cells. Data represent mean values (IC_{50}, μM) of three independent experiments ± standard deviations (S.D.).
[b] No mutations that confer resistance to amantadine.

Grade: 99%). The drug was dissolved in sterile ultrapure water and diluted in cell culture medium prior to use, and pH was adjusted to 7.2.

2.2. Viruses and cells

Six viruses in this study were isolated in dead or sick chicken from different poultry farms in Hebei Province of Northern China from 2001 to 2005 (see Table 1). Four isolates in 2005 were from the chicken vaccinated with commercially available licensed killed H5N2 vaccines. The isolates were typed as H5N1 influenza viruses by hemagglutinin inhibition (HI) and neuraminidase inhibition tests with a panel of reference antisera (Van Deusen et al., 1983). Our previous studies showed that these viruses caused 100% (8 of 8) mortality of 4-week-old SPF chicken within 2 days through intravenous infection with 0.2 ml of infectious allantoic fluid at 1:10 dilution (data not shown). On the basis of the criteria of viral virulence (Swayne and Halvorson, 2003), these viruses are highly pathogenic avian influenza viruses. These viruses were propagated in the allantoic cavaties of 10-day-old embryonated SPF chicken eggs and passed one time in Madin–Darby canine kidney (MDCK) cells. The virus stocks were stored in 0.5 ml aliquots at −80 °C until they were used in the amantadine sensitivity testing and the M gene sequencing. All manipulations of live viruses were conducted in biosafety level 3 facilities.

MDCK cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES buffer, the antibiotics penicillin G (100 IU/ml) and streptomycin sulfate (100 μg/ml) in a humidified atmosphere of 5% CO_2.

2.3. Amantadine sensitivity testing

To evaluate amantadine sensitivity, the plague reduction assay on MDCK cells was performed as described previously (Ilyushina et al., 2005; Hayden et al., 1980). Briefly, MDCK cells were seeded into 6-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for about 2 days. After the cells formed a monolayer, they were infected with different influenza viruses at a multipilicity of infection of 0.001 PFU/cell and incubated for 1 h at 37 °C. The cells were washed three times with PBS and then overlaid with DMEM containing 0.8% agar (AMRESCO, Lot: 3476B062), 2% FBS, and amantadine at different concentrations (0.01–100 μM). These were incubated at 37 °C for 48–72 h. Finally, monolayer cells were fixed in 10% formalin for 30 min and then stained with 0.1% crystal violet. Fifty percentage inhibitory concentration (IC_{50}) was calculated by probit analysis (Finney, 1971).

2.4. Sequencing and analysis of M genes

Viral RNAs were extracted from infected allantoic fluid with the TRIZOL LS Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Reverse transcription (RT)-PCR was performed using primers (primers are available upon request) specific for M gene segments as described previously (Widjaja et al., 2004). PCR products were purified and were cloned into the pGEM-T Easy Vector (Promega, USA), and then the DNA templates were sequenced on an ABI377 automated DNA sequencer using reagents from BGI Life Tech Co. Ltd. (China). The DNA sequences were compiled and edited by using the DNAMAN program (version 5.2.2). Nucleotide sequences studied in this article have been submitted to GenBank (accession numbers: EF175671, EF175672, EF175673, DQ351858, DQ351860 and DQ351859). The phylogenetic tree of M genes was generated with the MEGA program (version 3.1) by using the neighbor-joining method.

2.5. Investigation of amantadine use in poultry farms in Northern China

To make clear if the high incidence of amantadine-resistance was associated with widespread use of the drug, we randomly selected 10 chicken farms from the virus-isolated area in Hebei Province to investigate the amantadine use during October 2004 and September 2005. The annual data of amantadine use included the scale of chicken farms, route of administration, dosage and purpose.

3. Results

3.1. Susceptibility to the amantadine

The susceptibility of these influenza A (H5N1) viruses to amantadine was tested by plague reduction assay in MDCK

cells. Mean IC_{50} values were shown in Table 1. Of six H5N1 isolates, five were found to be resistant to amantadine, and one amantadine-sensitive isolate (A/Chicken/Hebei/718/2001) could be inhibited completely by amantadine at concentration as low as 0.044 μM. The IC_{50} values of the five resistant viruses ranged from 20.3 to 71.6 μM, which exceeded the IC_{50} for amantadine-sensitive viruses (1–10 μM) (Cheung et al., 2006). In this test, amantadine caused no cytotoxicity in MDCK cells at the range of concentrations tested (data not shown), which was consistent with previously published data (Ilyushina et al., 2006).

3.2. Molecular and phylogenetic analysis of M genes

Sequence analysis of M genes demonstrated that four of five resistant isolates carried the same single nucleotide mutation (AGT to AAT) resulting in an S31N substitution of M2 protein that confers resistance to amantadine, and one resistant isolate (A/Chicken/Hebei/706/2005) did not have any mutation that was found to related with amantadine-resistance (Table 1). In the phylogenetic tree of the M genes, when compared to the viruses available in the GenBank database, our three resistant isolates, closely clustered with a H5N1 virus (A/Chicken/Shanxi/2/2006) isolated in Northern China, formed an amantadine-resistant subgroup with other H5N1 influenza A viruses isolated in Asia (see Fig. 1).

3.3. Use of amantadine in poultry farms in Northern China

In China, Ministry of Agriculture has banned to sell and use amantadine in poultry farms (Jane, 2005). However, the drug was cheap and readily available, and so the chicken farmers frequently add amantadine into chicken food or water for the treatment and prophylaxis of lowly pathogenic avian influenza and other viral diseases (Table 2). The annual investigation from October 2004 to September 2005 showed that the mean days of medication were about 42 days in the small or large-scale chicken farms, indicating amantadine was used extensively in Hebei Province of Northern China.

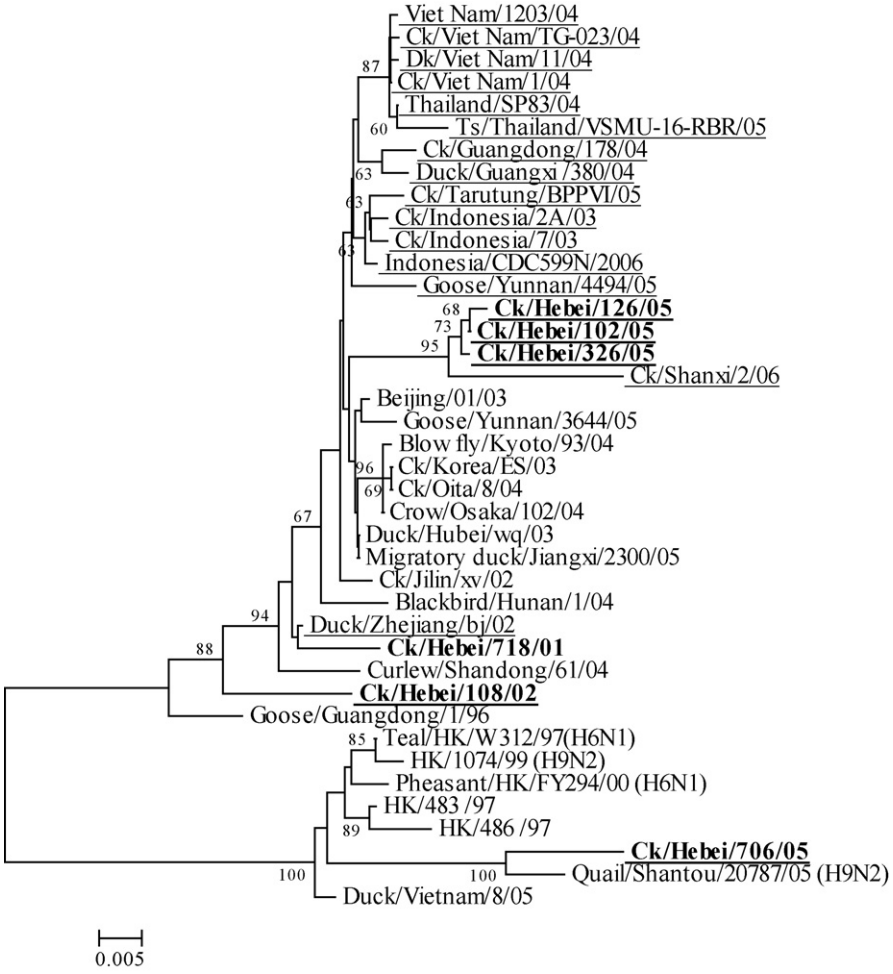

Fig. 1. Phylogenetic tree based on nucleotide sequences of M genes (nucleotide positions 1–982) of representative influenza A viruses isolated in Asia (that can be found in GenBank). The tree was generated by the neighbor-joining method with the MEGA 3.1 program and was rooted to midpoint. Numbers at branches are bootstrap values from 1000 replicates (less than 60% are not shown). A branch length scale bar is shown beneath the tree. The viruses in this study are shown in bold and amantadine-resistant variants are underline. *Abbreviations*. Ck, chicken; HK, Hong Kong; Ts, tree sparrow.

Table 2
Amantadine use in chicken farms in Northern China in 1 year (from October 2004 to September 2005)

Farms	No. of total chicken	Total days of medication in 1 year	Dosage (%; w/w)[a]	Routes of administration
A	8,300	37	0.03	Feedstuff
B	15,600	26	0.025	Feedstuff
C	10,400	43	0.022	Feedstuff
D	26,100	21	0.01	Drinking water
E	7,200	64	0.015	Drinking water
F	13,300	25	0.032	Feedstuff
G	4,300	63	0.01	Drinking water
H	21,700	38	0.012	Drinking water
I	5,400	42	0.025	Feedstuff
J	14,700	59	0.01	Drinking water

[a] Different proportions of amantadine were provided ad lib in feedstuff or drinking water.

4. Discussion

Amantadine, which was developed by an U.S. chemical company in 1964, has been used for the prophylaxis and treatment of influenza A virus for more than 30 years (Kitahori et al., 2006; Dolin et al., 1982). The use of amantadine has been associated with the rapid emergence of resistant viruses which are as genetically stable, virulent and transmissible as the wild-type virus (Bean et al., 1989; Fleming, 2003; Scholtissek and Webster, 1998). Recent studies have shown that the level of amantadine-resistant variants increased in H3N2 (Bright et al., 2005) and H5N1 influenza viruses (Ilyushina et al., 2005). In addition, amantadine-resistant H5N1 viruses were largely limited to be in Thailand, Vietnam, and Cambodia, while most H5N1 isolates from China and Indonesia are sensitive to amantadine (Hurt et al., 2007; Cheung et al., 2006; Guan and Chen, 2005). With this background, we can conclude that resistant H5N1 viruses were less commonly isolated in China than in other Asian countries.

To determine whether the same phenomenon occurred in Northern China, we assessed the susceptibility to amantadine of H5N1 influenza viruses isolated in Hebei Province between 2001 and 2005 in vitro test. Five (83.3%) out of six isolates were found to be resistant to amantadine, and only one isolate, A/Chicken/Hebei/718/2001, was confirmed as a sensitive strain (Table 1). These results indicated that the incidence of the amantadine-resistant viruses isolated in Northern China was particularly high.

It was reported that the genetic basis for resistance to amantadine was associated with amino acid substitutions at five positions 26, 27, 30, 31 and 34 in the transmembrane region of the M2 protein (Hay et al., 1985; Pinto et al., 1992). We analyzed M sequences of these H5N1 viruses, and found that four of five resistant isolates carried a specific amino acid S31N mutation related to amantadine-resistaince in the M2 protein (Table 1). Interestingly, one H5N1 virus (A/Chicken/Hebei/706/2005), confirmed as a resistant strain in vitro test, did not carry specific mutations that could confer resistance to amantadine. From this result, we could see a discrepancy between this study and previous findings. Recently it was shown that changes in HA of avian virus could confer the resistance to adamantanes (Ilyushina et al., 2007). Further studies should be conducted on whether a drug-resistant phenotype was influenced by other mutations in HA and M2 protein of the A/Chicken/Hebei/706/2005 virus by using other methods (such as reverse genetics).

In the phylogenetic tree of the M genes, when compared to the viruses available in the GenBank database, our three amantadine-resistant isolates and other resistant H5N1 viruses isolated in Asia dispersed a relatively localized area to form an amantadine-resistant subgroup (see Fig. 1). This subgroup has the characteristic S31N amino acid mutation in the M2 protein. The clustering of the resistant viruses suggested a common origin of these viruses, even though they were isolated in several countries at different times, which was consistent with previously published data (Barr et al., 2007). The origin of the subgroup is unexplained by far. Interestingly, the M gene of A/Chicken/Hebei/706/2005 fell into another clade clustering with Quail/Shantou/20787/05 (H9N2), which indicated that this H5N1 amantadine-resistant virus could be a recombinant virus. We need further to elucidate the relationship between our isolates and other resistant strains isolated in other countries, and elucidate the phylogenetic relationship between their M genes and the HA genes.

It is generally accepted that amantadine-resistant viruses emerge under the pressure of the drug (Masuda et al., 2000; Harper et al., 2005). Since 2004, China has enforced a large-scale compulsory vaccination programme (Cyranoski, 2005). However, in the field of influenza vaccination, neither commercially available nor experimentally tested vaccines have been shown so far to achieve the ideal goals (Lee and Suarez, 2005). One research showed that incomplete protection at the flock level, even if a vaccine is effective in individual birds, can result in ‘silent spread’ (Savill et al., 2006). Extensive surveillance has revealed that H5N1 viruses have become predominant and then endemic in vaccinated poultry in Southern China (Smith et al., 2006). Therefore, this ‘silent infection’ may make the vaccinated birds which appear healthy be infected and excrete the field virus ‘under cover’ of the vaccine. In this situation, if amantadine was used extensively, influenza viruses in vivo would have the chance to contact this antiviral agent for a long times, and drug-resistance could occur easily by passing the virus in the presence of amantadine. In our research, most of amantadine-resistant viruses were isolated from vaccinated chicken in Hebei Province of Northern China, and amantadine was used exten-

sively in poultry farms in this area (Table 1). With widespread use of amantadine in these chicken farms, amantadine-resistant variants could be easily induced, which maybe was one of reasons of particularly high amantadine-resistance incidence in H5N1 viruses in our study. Our data indicated that it will be important to further monitor amantadine-resistant H5N1 strains circulating in animals and humans.

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