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Increased adamantane resistance in influenza A(H3) viruses in Australia and neighbouring countries in 2005

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Abstract

The prevention and control of disease caused by seasonal and potential pandemic influenza viruses is currently managed by the use influenza vaccines and antivirals. The adamantanes (amantadine and rimantadine) were the first antivirals licensed for use against influenza A viruses and have been used extensively in some countries. Since the early 2000s increased resistance to these drugs has been reported especially in the A(H3) viruses. In this study we analysed recent human influenza A strains isolated in Australia and regionally for evidence of resistance to adamantanes and found evidence of significant resistant emerging during 2005.

Keywords: Amantadine; Rimantadine; Matrix gene; Resistance; Australia; South East Asia

1. Introduction

Influenza viruses cause a significant level of morbidity and mortality in the population every year and effects countries in temperate and tropical regions (Nicholson et al., 2003). While vaccination programs are the major public health measure used to ameliorate the effects of infection, especially in the elderly, outbreaks still occur in nursing homes, schools and cruise liners (DeStefano, 1982; Drinka et al., 1999; CDC, 2001). Another treatment or preventative measure is the use of influenza antiviral drugs. There are two classes of drugs, which can be used, the older group known as adamantanes consisting of amantadine (Symmetrel™) and rimantadine (Flumadine™) (Aoki, 1998) and the newer group known as neuraminidase inhibitors (NI's) (Gubareva et al., 2000) consisting of oseltamivir (Tamiflu™) and zanamivir (Relenza™). While the adamantanes only work against influenza A viruses and have some unwanted side effects (Aoki, 1998) their lower price has ensured their continued use especially in vulnerable aged adults (Colgan et al., 2003).

The adamantanes work by blocking the ion channel formed by the M2 protein of influenza A viruses which inhibits the early stages of virus replication (Hay, 1992; Pinto et al., 1992; Aoki, 1998). However, the emergence of resistance following treatment with the adamantanes has been an issue in their use (Belshe et al., 1989) although resistance usually appears only transiently, following treatment and has not resulted in significant spread or maintenance of resistant strains in circulation (Ziegler et al., 1999). The levels of circulating resistant viruses have generally been less than 1%, although they can be much higher in groups undergoing treatment (up to 80%) when they are carefully monitored (Shiraishi et al., 2003). These levels however, have not persisted in the population and wild type viruses have continued to predominate. The mechanism of resistance is well understood and revolves around mutations in the M2 protein which forms the ion channel and leads to loss of binding of these drugs (Hay et al., 1985; Aoki, 1998,). Several amino acid substitutions (at positions 26, 27, 30, 31 and 34) in the M2 protein, prevent binding of the adamantanes or change the structure of the ion channel to allow it to operate even in the presence of bound drug, both types of change result in the generation of resistant viruses (Hay

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et al., 1986; Astrahan et al., 2004). In contrast, resistance to the NI's has been very low, especially for zanamivir and to a lesser extent oseltamivir (McKimm-Breschkin, 2000).

A recent publication by Bright et al., 2005, has highlighted an increasing and persistent level of resistance to adamantanes in influenza A(H3) viruses, especially in China and Hong Kong (SAR of China) in 2003–2004. China for example, saw levels of adamantane resistance in influenza A(H3) viruses rise from below 10% during 1995–2002, growing rapidly to 57.5% in 2003 and then rising up to 73.8% in 2004 (Bright et al., 2005). These increased levels of resistance in A(H3) viruses have been reported in other countries, such as the United States where levels of resistance have grown rapidly in the last few years from below 2% to 15% in 2004 (Bright et al., 2005) rising to 92.3% during the 2005–2006 influenza season (Bright et al., 2006). Interestingly while influenza A(H1) viruses tested between 1998 and 2004 appeared to be almost universally sensitive to the adamantanes, recent data shows that these too are becoming resistant with 25% of A(H1) viruses from the US collected early in the 2005–2006 influenza season, containing the molecular changes which confer resistance (Bright et al., 2006). In this study we examined influenza A viruses isolated from patients in Australia, New Zealand, South East Asia, Macau (SAR of China) and Taiwan (Province of China), over a number of years for resistance markers to adamantanes. We also compared the use of amantadine in Australia (rimantadine is not licensed in Australia) with the levels of resistance. Adamantane resistant and sensitive viruses were also examined for their sensitivity to the licensed NI's, zanamivir and oseltamivir and their phylogenetic relationships, based on their haemagglutinin and matrix genes, were also investigated.

2. Materials and methods

2.1. Viruses

Influenza A(H3) and A(H1) viruses were received from WHO National Influenza Centres, WHO Influenza Collaborating Centres and other regional laboratories and hospitals from Australia, New Zealand, and the Asia/Pacific region. Viruses were received as isolates passaged in cell culture or as original clinical samples in which influenza A had been detected by immunofluorescence or by RT-PCR. Once received at the Centre, the isolates were cultured in MDCK cells and monitored for growth by CPE and the presence of haemagglutination activity using turkey red blood cells (RBC's) as previously described. Positive samples were typed using the haemagglutination inhibition assay (HAI) against a panel of known standard reference viruses and their homologous ferret antiserum (CDC, 1982).

2.2. Sequencing and phylogenetic analysis

RNA extraction, RT-PCR and sequencing were performed as previously published (Barr et al., 2003). Sequences were assembled using the Lasergene Seqman package IV (DNAStar 6) and phylogenetic relationships determined with PHYLIP V 3.5.7 (Felenstein, 1989) using the neighbour-joining method on ANGIS (Australian National Genomic Information Service) and dendograms were drawn using Treeview (Page, 1996). Bootstrap confidence values were calculated using 100 replicates before determining phylogenetic distances with PHYLIP.

2.3. Adamantane sensitivity assay

A biological assay to determine virus susceptibility to both rimantadine and amantadine was completed following previously published methods (Bright et al., 2005), except that viruses were tested using three virus dilutions (1:2, 1:20, and 1:200) and plates were incubated at 35 °C for 24 h, rather than 37 °C for 36 h.

2.4. Neuraminidase enzyme inhibition assay

A fluorescence-based neuraminidase (NA) enzyme inhibition assay was used to assess susceptibility of viruses to the neuraminidase inhibitors zanamivir and oseltamivir carboxylate as described previously (Hurt et al., 2004).

3. Results

3.1. Levels of adamantane resistance in Australia and regionally

A total of 102 A(H3) viruses and 37 A(H1) viruses isolated at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne in 2005, were examined for the specific mutations known to correlate with resistance to the antiviral drugs amantadine and rimantadine (Table 1). These were selected from 1329 A(H3) and 374 A(H1) viruses received at the Centre in 2005, with sequenced viruses therefore representing 7.7% and 9.9% of the total number received. The average age of the patients from which the viruses were examined was 16.6 years with a range of 3 months to 77 years (ages were only available for 81/139 patients). In 2005, 42% (43/102) of influenza A(H3) viruses and 0% (0/39) A(H1) viruses had substitutions

Table 1
Geographic origin and proportion of A(H3) resistant viruses in 2005

Country	2005 A(H3) viruses		2005 A(H1) viruses	
	No. resistant/no. tested	% resistant	No. resistant/no. tested	% resistant
Macau (SAR)	10/10	100	0/3	0
Taiwan	1/2	50	0/1	0
Thailand	3/13	23	0/2	0
Malaysia	8/10	80	0/3	0
Cambodia	2/2	100	–	–
Singapore	6/12	50	0/1	0
Philippines	2/10	20	-	–
Indonesia	0/3	0	0/1	0
Australia	7/22	32	0/23	0
New Zealand	3/12	25	0/2	0
South Africa	1/5	20	0/1	0
Sri Lanka	1/1	100	–	–
Total	43/102	42	0/37	0

which would be expected to confer resistance to adamantanes. The highest levels of resistance were noted in A(H3) viruses from Macau (SAR) 100% (10/10), Singapore 50% (6/12) and Malaysia 80% (8/10), while Australia had 32% (7/22). The levels of resistance in influenza A(H3) viruses isolated between 2000 and 2004 period were much lower with 0% of Australian viruses (0/62 total; 0/12 in 2000, 0/11 in 2001, 0/10 in 2002, 0/15 in 2003, 0/14 in 2004) and only 3.6% from viruses isolated elsewhere (1/26; with the one resistant virus being a 2003 Malaysian isolate) showing resistance markers for the adamantanes. Analysis of the isolation dates for Australian A(H3) resistant viruses showed that they first appeared in late May 2005 in the Northern Territory and then later in other states accompanying the peak period of influenza in Australia. Testing of a small number of influenza A (H1) virus collected pre-2005 from the region also failed to detect any with adamantane resistance (0/6). All resistant viruses had the same single nucleotide change (AGT to AAT) resulting in an S31N substitution in the M2 protein.

3.2. Use of amantadine clinically in Australia and resistance levels

The level of resistance to adamantanes was compared to the sales of Symmetrel™ (amantadine) in Australia over the 2001–2005 period. While the main use of Symmetrel™ in Australia appears to be for the treatment of Parkinson's disease, no records of its use for this purpose versus influenza treatment or prophylaxis are available. Symmetrel™ unit (14× 200 mg tablets) sales in Australia were virtually constant over the period (2001; 13,680 units sold, 2002; 14,049; 2003; 14,414; 2004; 15,113; 2005; 14,777) and did not correlate with the increased levels of resistance seen in Australia in 2005 (Personal communication, Ben Guthrie, Novartis, Australia).

3.3. Bioassays for adamantanes and neuraminidase inhibitors

A random selection of 10 A(H3) viruses with the S31N M2 substitution and 10 A(H3) viruses showing no substitutions, were tested in a bioassay with amantadine and rimantadine. The viruses with the S31N substitution grew well in the presence of either drug with no reduction in HA titre following growth in up to 20 μg/ml of drug (compared to growth of the viruses without drug), while the viruses with no substitutions were inhibited by concentrations as low as 0.2 μg/ml resulting in a significant reduction in haemagglutinin titre. The bioassay results correlated 100% with the sequencing results, with all S31N viruses tested being resistant to both amantadine and rimantadine. The viruses used in the bioassay were also tested for sensitivity to the NI's along with other viruses. None of the amantadine resistant influenza A(H3) viruses ($n = 20$) had a reduced sensitivity to the NI's and had a mean (± S.D.) IC_{50} of 0.85 (±0.26) nM for zanamivir and 0.35 (±0.13) nM for oseltamivir carboxylate (the active form of Oseltamivir) compared to a mean IC_{50} of 0.96 (±0.51) nM for zanamivir and 0.31 (±0.10) nM for oseltamivir for the amantadine sensitive viruses ($n = 20$). Similar figures were obtained for the influenza A(H1) amantadine sensitive viruses tested (results not shown). These NI IC_{50} levels would indicate there was no increased resistance to either zanamivir or oseltamivir in the amantadine resistant influenza A(H3) viruses.

3.4. Phylogenetic analysis of the haemagglutinin and matrix A(H3) genes from recent isolates

The phylogenetic relationships of the matrix gene and the HA1 domain of the haemagglutinin gene were also compared for the 2005 A(H3) viruses. All of the 2005 influenza A(H3) adamantane resistant viruses grouped into a single subgroup based on their Matrix gene (Fig. 1a) indicating a possible single precursor virus. This pattern was also seen with the phylogenetic analysis of the HA1 domain of the HA gene (Fig. 1b) with the resistant A(H3) 2005 viruses. There the adamantane resistant viruses fell into a single HA1 clade that first emerged in 2004, but now contains the majority of the recent isolates. This clade has the characteristic S193F and D225N amino acid changes in the HA1 domain compared to other recent A(H3) viruses. From contemporary influenza A(H3) viruses only one adamantane resistant strain (A/Malaysia/434/2003) fell into another clade but there were a few recent sensitive strains that fell within the S193F/D225N clade (A/Thailand/151/2005 and A/Newcastle/4/2005) indicating that these specific HA changes were not directly linked to the M2 changes that confer adamantane resistance. The Genbank accession numbers for the HA1 sequences shown in Fig. 1a are DQ865945, DQ865946, DQ865947, DQ865948, DQ865949, DQ865950, DQ865951, DQ865952, DQ865953, DQ865954, DQ865955, DQ865956, DQ865957, DQ865958, DQ865959, DQ865960, DQ865961, DQ865962, DQ865963, DQ865964, DQ865965, DQ865966, DQ865967, DQ865968, DQ865969, DQ865970, DQ865971, DQ865972, DQ865973, DQ865974 and for the Matrix gene sequences shown in Fig. 1b are DQ849002, DQ849003, DQ849004, DQ849005, DQ849006, DQ849007, DQ849008, DQ849009, DQ849010, DQ849011, DQ849012, DQ849013, DQ849014, DQ849015, DQ849016, DQ849017, DQ849018, DQ849019, DQ849020, DQ849021, DQ849022, DQ849023, DQ849024.

4. Discussion

Despite the introduction of neuraminidase inhibitors, amantadine and rimantadine continue to be used for the treatment or prevention of influenza A, mainly due to their low cost. Following recent reports of increased resistance of influenza A(H3) viruses to adamantanes in Asia and in North America (Bright et al., 2005, 2006), an analysis of strains isolates in Australia and regional countries was undertaken, to determine the level of resistance in this region. In 2005, resistant strains were common in Australia (32%) and several Asian countries, Malaysia, Thailand, Chinese SAR Macau and Singapore (ranging from 50% to 100% resistant strains), while virtually no resistance was detected in strains isolated in or before 2004. While the numbers of A(H3) viruses are not large for Australia A(H3) viruses

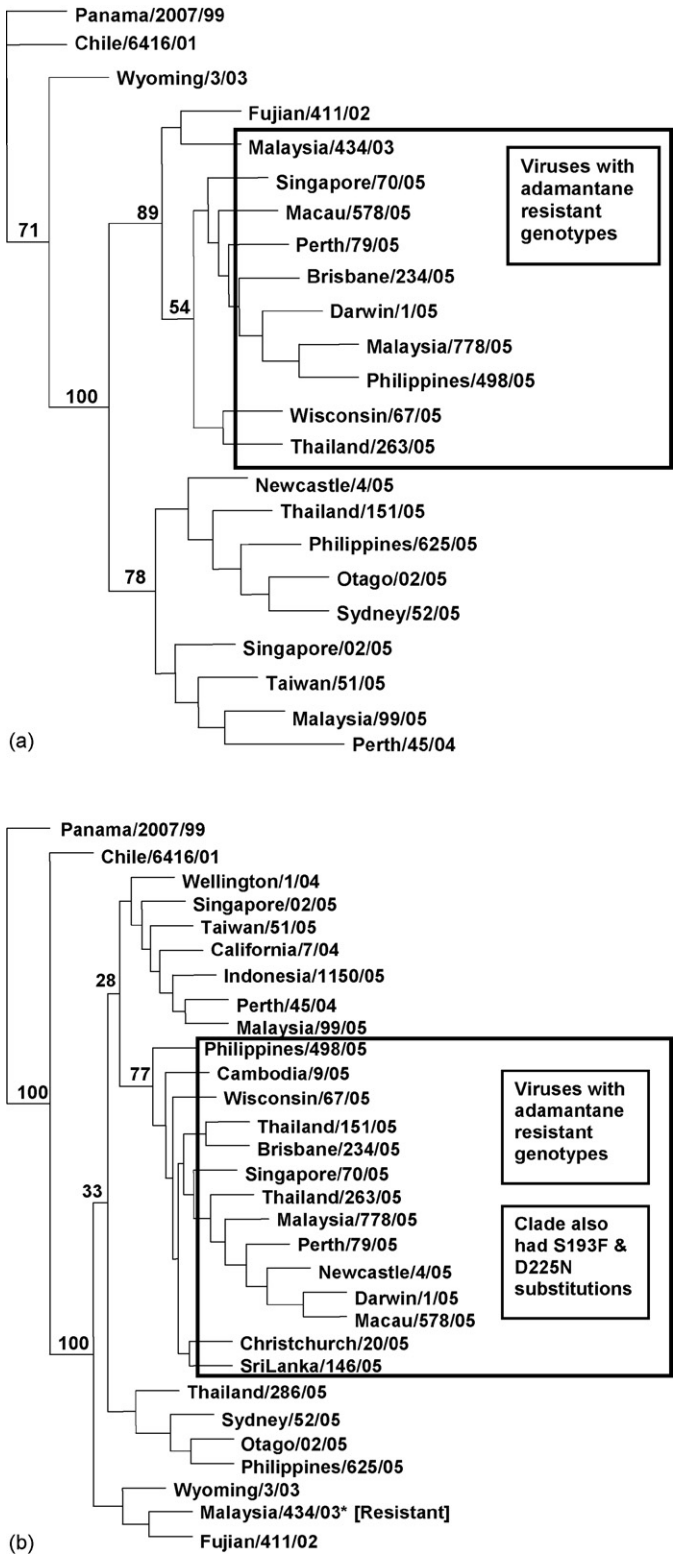

Fig. 1. Phylogenetic analysis of the influenza A(H3) matrix gene (a) and the HA1 domain of the haemagglutinin gene (b) from selected viruses. Bootstrap values from 100 replicates are shown for selected nodes. Viruses showing genetic changes conferring resistance (S31N in M2) are boxed on both figures. All other viruses had a sensitive genotype except for A/Malaysia/434/2003, which was also resistant (S31N).

were sampled from all 7 geographically distant states and for other countries generally represent all 2005 viruses available at the Centre for testing. It is known that a substitution of any one of five amino acids in the M2 protein in influenza A viruses can confer resistance to amantadine or rimantadine (Hay et al., 1985; Hay, 1992; Aoki, 1998), however in this study only one substitution (S31N) was evident in all of the influenza A(H3) viruses that were isolated in 2005 that had a resistant phenotype. Other reports have confirmed that this is by far the most common substitution seen recently in influenza A(H3) viruses in man with 98.2% of resistant viruses in one study (Bright et al., 2005) and 100% in another (Bright et al., 2006) only this change. In contrast a study of recent A(H5N1) viruses from SE Asia, found the S31N change was almost invariably associated with an additional L26I substitution (Cheung et al., 2006).

With the advent of high levels of adamantane resistance in influenza A(H3) strains in the United States during the 2005–2006 influenza season, CDC issued a recommendation not to administer the adamantanes to people with influenza or to use it prophylactically (CDC 2006). CDC recommended the use of NI's because these drugs have not been associated with any significant level of resistance to date (MMWR 2006), a finding confirmed in this study. Possible reasons for increased resistance to adamantanes might be due to the increased use of adamantanes in the region as resistant viruses are quickly generated in individuals under treatment (Aoki, 1998). It is likely that resistant strains of A(H3) were introduced into Australia by travellers, as the use of Symmetrel™ (amantadine hydrochoride) is low and while licensed for use for the treatment and prophylaxis of influenza is mostly used for Parkinson's disease. Furthermore there has been no significant increase in sales over the period 2001–2005. Rimantadine is not sold or licensed in Australia.

A recent study of avian influenza A H5, H6, H7, H9 subtypes also showed an increased level of resistance in H5, H7 and H9 subtypes during the 2000–2004 period (Ilyushina et al., 2005). An analysis of avian influenza H5, H7 and H9 strains collected during the period 1979–1983, failed to detect any resistant strains (0/43) from North America or South East Asia (0/20). By contrast, in the period 2000–2004 9/120 (7.5%) of North American viruses and 56/169 (33%) of viruses from South East Asia exhibited resistance. Most of the resistant viruses were of the influenza A(H5) subtype. No H6 adamantane resistant viruses were reported. Only the A(H5) resistance to adamantane had increased over this period and then only in the SE Asia derived viruses while the H7 and H9 resistance levels had reduced to background by 2004. The increased resistance of some avian influenza viruses to amantadine has been suggested to be caused by excessive use of the drug by farmers in China (MacKenzie, 2005). Resistance to adamantanes in A(H5) viruses isolated from humans in Vietnam, Cambodia and Thailand in 2004–2005 has also been found in the majority of isolates (WHO, 2005).

It is interesting to speculate on why the levels of resistance of recent A(H3) viruses are higher than influenza A(H1) viruses isolated during the same period. A previous study also showed a higher prevalence of resistant influenza A(H3) viruses 22/66

(33%) than influenza A(H1) viruses, 9/45 (20%) in separate outbreaks over two influenza seasons (Saito et al., 2003). Differential resistance patterns with influenza A subtypes has also been reported with avian viruses, with influenza A(H5) showing increased resistance but influenza A(H6) viruses not showing any resistance to date (Ilyushina et al., 2005). None of a selection of 20 of the A(H3) resistant viruses isolated in 2005 in this present study showed increased levels of resistance to the two licensed NI antivirals, oseltamivir or zanamivir in a bioassay. Phylogenetically all of the resistant human influenza A(H3) 2005 resistant viruses were similar, having the identical M2 substitution S31N, and grouping together phylogenetically for both the matrix gene and the HA1 gene of haemagglutinin (where all the 2005 viruses had the characteristic S193F and D225N substitutions). The tight grouping of these resistant viruses based both on the M2 gene and the HA1 domain of the HA gene, gives weight to a common origin of these viruses, even though they are now found in several countries. Interestingly two 2005 A(H3) viruses (A/Newcastle/4/2005 and A/Thailand/151/2005) which fell into the same HA1 clade, were sensitive to adamantanes, indicating a possible reversion to a sensitive genotype in these viruses. It may also only be a matter of time before the majority of A(H1) viruses develop a high level of resistance to adamantanes as resistance appears to be currently increasing in this subtype (Bright et al., 2006).

Clearly influenza A(H3) viruses with the S31N substitution are capable of normal spread within the community with no viral fitness compromises and may in fact now have a selective advantage. This has not been the case in the past, as studies in the UK, Japan and elsewhere have indicated that circulation of adamantine resistant viruses are rare (Ziegler et al., 1999; Dawson, 2000) even when the drug useage has been significant, such as Japan in the late 1990's (Masuda et al., 2000). Resistance levels however do rise dramatically in infected patients treated with amantadine (Shiraishi et al., 2003). These levels maybe even higher if viral sequences were examined using cloning techniques to examine individual viruses instead of the usual procedure of sequencing samples of whole virus isolates, that may contain mixed populations of drug resistant and sensitive viruses. This approach has been used in a recent study by Kiso et al., 2004, to analyse influenza A(H3N2) viruses in Japanese children before and after treatment with the NI oseltamivir. They found evidence for increased levels of resistance compared to previous reports that used standard sequencing techniques. Reducing the level of resistance of circulating influenza A(H3) and A(H1) viruses to adamantanes would be useful as many of the circulating influenza A(H5) viruses especially in China and Indonesia are still sensitive to adamantanes (Cheung et al., 2006) and so these drugs could still be useful if one of these sensitive A (H5) viruses evolved through a recombination event into a human-to-human transmissible pandemic strain. There is also the possibility of using the adamantanes and NI's in combination (Ilyushina et al., 2006) to combat pandemic influenza and the WHO has also recently recommended the use of amantadine and rimantadine against H5N1 viruses in humans if NI's were not available and the viruses were known or likely to be susceptible to these drugs (WHO, 2006). To ensure the best use of all influenza antiviral drugs against seasonal and potential pandemic viruses it is therefore important to monitor the level of resistance for both the M2 inhibitors and NI's, in all circulating subtypes of influenza A in both human and avian species.

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