ければならない。さらに、一般に情報に関して言えば、迅速さと詳細さは基本的に相容れない。しかし、本分担研究班で対象としている臨床情報共有システムはその双方を満たしている必要がある。すなわち、臨床医にとって新型インフルエンザ患者の診断や治療に役立つ情報を、より迅速に提供する必要がある。

迅速さと詳細さを両立させるためにはどうすればよいか。この点を中心に、日本で新型インフルエンザ患者が発生した段階で真っ先に患者を診察し治療にあたるであろうと思われる、第一種・第二種感染症指定医療機関で感染症を担当する医師を中心とした会議および聞き取り調査を行い、臨床レベルで必要とされるサーベイランスについて検討を行なった。

その結果、前述のような意見集約を行うことができた。

まず、患者数が少ない時点（おそらく全国で 10 例～20 例程度）では、多くの臨床医がかなり詳細な情報を知りたいと思うであろう。臨床像、抗ウイルス薬の効果、予後など、項目は様々である。この時期に関してデータ収集項目をあらかじめ決定しておくことはおそらく困難であり、その意味では項目もその記述内容も自由に変更できる形式が望ましい。**資料 2** の叙述および臨床経過表示形式がそのひな形となるであろう。

一方、患者数が多くなってくると、このような詳細な情報を記入して提供することの意義が段々薄れていくであろう。提出する方も労力を要し、集計にも時間を要するので、迅速なフィードバックができない。その点では、**資料 1** のような簡単な書式に短時間で記入して送付できる形式が望ましい。

送付方法として、最も安全なのはおそらくメール添付であろう。郵便、FAX はしばしば誤送信や不達がある。メールが最も確実かつ迅速に情報を届ける手段である。但し、送信者がメールアドレスを間違えないなど、最低限の注意は必要となるが、これは FAX の間違い番号や郵便の住所記述間違いでも同じことが起こるので、メール固有の問題ではない。さらに安全な手段として、専用の Website を経由して提出する手段がある。これは一見良さそうであるが、初期投資および維持管理に費用がかかり、また修正するにも費用がかかるなど、費用対効果を考えると良い選択とは思えない。

IT の側面ではむしろ、これらの情報を管理するサーバを設け、そこに全員がアクセスできる形でのデータ共有システムを構築することが必要である。新型インフルエンザが発生し、臨床医たちが診療に忙しくなると、深夜や空いた時間に簡単にデータを見られるシステムが必要である。情報は日々蓄積されるので、頻繁にかつ簡便に広範囲の情報にアクセスできるとなると、この方法が最もよいと考えられる。

今回は議論できなかったが、提出されたデータは基本的にこのシステムに参加する人々の間でシェアできるような形が望ましい。一方、誰でもアクセス可能な情報としては、疫学情報（例：死亡率、抗ウイルス薬の投与が有効であった症例の割合など）のみに絞った方が、善意でデータを出す側の立場に立った情報共有と言える。

今後の課題としてはいくつかの点が挙げられる。

（1）資料1と資料2の二つの方法でデータ収集を行うとすると、どの時点で切り替えるか、切り替えの前後で混乱しないか

（2）今回は、東京都内および周辺の第一類・第二類感染症指定医療機関の医師を中心に意見収集を行なったが、このメンバーはすでに感染症指定医療機関のグループを形成している。東京都内および周辺以外の感染症指定医療機関のスタッフのグループも他にたくさんあるように思われるので、今後どのようにそれらを掌握し、巻き込んでいくか

（3）各施設から収集されたデータの帰属、すなわち、国のデータであるならば、公衆衛生的視点から、個人情報保護法、情報公開法などとの整合性を考慮する必要がある

E. 結論

新型インフルエンザが発生した際に、臨床実地で生かすことのできる臨床経過情報共有システムに関する検討を行った。症例数が少ない段階では叙述形式の様式、症例数が増えてきたら疫学情報を得るためのラインリスティング形式によるデータ収集が望ましいと考えられた。データは電子ファイル化し、メール添付で集計先に送付する。集計先では迅速にこれを集計し、サーバ上に置いて誰もが見られるような形式が望ましい。この実現のためには、データ公開の範囲などさらなる検討事項がある。

F. 研究発表

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なし

G. 知的所有権の出願・登録状況

特記すべきものなし

研究協力者（順不同）
角田隆文（荏原病院）
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資料1，新型インフルエンザ患者臨床情報共有データベース

	管理情報		患者基本情報												
症例通し番号	データベース編入日	報告者ID	患者ID	年齢	性別	患者在住都道府県・市町村	感染したと推定される日	感染経路	発症日	初診日	入院日	死亡の有無	死亡日	剖検の有無	退院
1															
2															
3															
4															
.															
.															

	臨床症状							検査							
症例通し番号	38度以上の発熱	乾性がいそう	湿性がいそう	呼吸困難	肺炎	気管内挿管による人工呼吸	下痢	ラボ診断(PCR)によるH5N1の確認	好中球減少(1500以下)	胸部X線写真上の異常陰影	胸水				
1															
2															
3															
4															
.															
.															

	治療						その他								
症例通し番号	抗インフルエンザウイルス薬投与	そのドース	開始日	臨床効果	その他の薬剤投与	その臨床効果	参考情報								
1															
2															
3															
4															
.															
.															

資料２　新型インフルエンザ臨床経過表

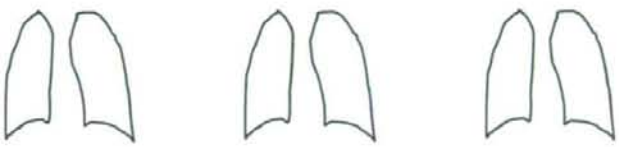
1．氏名、年齢、性別、etc.
2．主訴　□発熱、□呼吸困難、□咳嗽、etc
3．既往歴、etc
4．現病歴
5．入院時身体所見：体温、脈拍、血圧、呼吸数
6．入院後経過

日数	1	2	3	4	5	6	7	8	9					
体温														
38℃														
37℃														

日数	1	2	3	4	5	6	7	8	9					
検査														
WBC	3.0	3.5	10	12	8.5	5.0								
CRP	6.0													
AST														
etc														

日数	1	2	3	4	5	6	7	8	9					
治療														
ﾀﾐﾌﾙ														
ﾒﾛﾍﾟﾝ														

画像



Ⅲ. 研究成果の刊行に関する一覧表

別紙4

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

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ORIGINAL ARTICLE

Molecular evolution of human influenza A viruses in a local area during eight influenza epidemics from 2000 to 2007

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Abstract A total of 1,041 human influenza A virus isolates were collected at a clinic in Niigata, Japan, during eight influenza seasons from 2000 to 2007. The H3N2 subtype accounted for 75.4% of the isolates, and the rest were H1N1. Extremely high rates of amantadine-resistant strains of H3N2 subtype were observed in 2005/2006 (100%) and 2006/2007 (79.4%), while amantadine-resistant strains of H1N1 subtype were only detected in 2006/2007 (48.2%). Sequence and phylogenetic analysis of the HA1 subunit of the hemagglutinin (HA) gene revealed a characteristic linear trunk in the case of H3N2 viruses and a multi-furcated tree in the case of H1N1 and showed a higher sequence diversity among H3N2 strains than H1N1 strains. Mutations in the HA1 from both subtypes were mainly found in the globular region, and only one-third of these were retained for two or more successive years. Higher diversity of H3N2 viruses was mainly attributable to a higher fixation rate of non-synonymous mutations and to a lesser extent to a higher nucleotide substitution rate than for H1N1. Our analysis showed evidence of four positively selected sites in the HA1 of H1 and five sites in that of H3, four of which were novel. Finally, acquisition or loss of *N*-glycosylation sites was shown to contribute to the evolution of influenza A virus, especially in the case of H3N2, which had a higher tendency to acquire new glycosylation sites.

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Introduction

Type A influenza viruses are major pathogens for humans. Annual influenza epidemics are estimated to affect around 3–5 million of the world's population. During the last century, four influenza pandemics alone claimed more than 50 million lives [35]. Vaccination remains the primary measure for mitigating the outcomes of annual influenza epidemics, but vaccine strains have to be updated every year due to the continuous evolution of the viral proteins [6]. Antiviral drugs like M2 channel inhibitors (amantadine and rimantadine) and neuraminidase inhibitors (oseltamivir and zanamivir) provide an alternative for controlling influenza infections, although high resistance rates to the former have widely limited its use [10, 17, 28].

Influenza A viruses are negative sense single-stranded RNA viruses belonging to the family *Orthomyxoviridae* and possess a genome of eight single-stranded segments. Influenza A virus is further classified based on the antigenic properties of its surface glycoproteins, the haemagglutinin (HA) and the neuraminidase (NA). Influenza A/H3N2 and A/H1N1 subtypes are the major subtypes currently circulating in human populations [24, 37]. HA is of special interest due to its role in the viral entry mechanism and immune recognition. It consists of two subunits: HA1, which contains the receptor-binding and antigenic domains, and the HA2 subunit, which is responsible for the fusion of the virion with the endosomal membrane in the host cell [40, 41]. The HA1 subunit undergoes a process

termed positive Darwinian selection through continuous antigenic mutations that allow the virus to evade the host's humoral immune response [12].

Since the last decade, there has been an explosive increase in sequence data for influenza virus. The availability of high-throughput molecular biology and computational technologies and software provides powerful tools for phylogenetic and evolutionary analyses. Many studies have analyzed the evolution of the HA1 subunit of influenza A/H3N2 viruses and fewer have examined that of A/H1N1 [24]. However, in most of these studies, geographically dispersed isolates were analyzed using routine phylogeny, and issues like evolution rate and positive selection were explored in only a few studies [4, 5, 13, 25]. Moreover, the aim of surveillance programs usually focuses on identifying serologically novel strains and determining their genetic variation, which might bias the results of studies in which the evolution of influenza virus is analyzed [43]. In this study, we analyzed and compared the genetic diversity and mechanisms underlying the evolutionary dynamics of the HA1 of both influenza A/H3N2 and A/H1N1 viruses isolated in a single area in Niigata, Japan, from 2000 to 2007, using evolutionary models and analytical bioinformatics tools [26, 45, 46].

Materials and methods

Sample collection and isolation

During eight influenza seasons from January 2000 to April 2007, nasopharyngeal swabs were obtained from patients with influenza-like illness symptoms at a pediatric clinic in Niigata City, the capital of Niigata prefecture, Japan. The clinic is located in the central part of the city with ~2000 outpatient visits per month. All of the patients from whom swabs were obtained were local inhabitants, with the majority of them residing within a 10-km radius of the clinic. None of the patients in this study received anti-influenza (amantadine) treatment before the swab was obtained. Swabs were checked by using an influenza rapid diagnostic kit such as QuickVue Rapid SP influ (DS Pharma Biomedical Co., Ltd, Osaka), Espline Influenza A&B–N (Fujirebio Inc., Tokyo), or Quick S-Influ A/B "Seiken" (Denkaseiken Co., Ltd, Tokyo). Another swab was obtained from each influenza A-positive patient upon signing an informed consent statement. Swabs were suspended in viral transport medium and kept at 4°C until transportation to our laboratory within 1 week. For influenza virus isolation, 100-µl aliquots of supernatant of nasopharyngeal swabs were inoculated onto MDCK cells and then kept at 34°C under 5% CO_2 until a specific cytopathic effect was detected. The viruses were passaged three times to obtain sufficient virus titers for virus identification. Isolates were then antigenically subtyped by hemagglutination inhibition test [8]. Virus isolates were then stored at −80°C until further analysis.

RNA extraction, PCR, and nucleotide sequencing

One-hundred-microliter aliquots of the supernatant after the third culture passage were used for viral RNA extraction with an Extragen II kit (Kainos, Tokyo, Japan), according to the manufacturer's instructions. RNA was reverse transcribed to complementary DNA with the influenza A virus universal primer Uni12, as described elsewhere [18]. PCR was performed with M gene-specific primers to amplify the M2 fragment, a 231-bp product covering nucleotides 680–910 [23]. The PCR products were sequenced to examine amino acid mutations at positions 26, 27, 30, 31, and 34, which confer resistance to amantadine [2, 15]. Following the initial characterization, representative viruses from each subtype were selected at random, but distributed over the epidemic of each season (2–4 isolates each from the beginning, middle, and end of the epidemic in each season) to avoid capturing samples from one outbreak. In the case of the 2006–2007 season, when both amantadine-sensitive and amantadine-resistant H3N2 and H1N1 viruses co-circulated, the same selection criteria was used, but this time relevant numbers of samples from both sensitive and resistant lineages were selected. The HA1 fragments of the selected samples of H3N2 and H1N1 viruses were amplified with specific primers [3]. The PCR products were purified using an MSB spin PCRapace purification kit (Invitek, Berlin, Germany), labeled by the use of a BigDye Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, Foster, CA) according to the manufacturer's instructions, and then analyzed using an ABI 3100 automatic DNA sequencer.

Sequence alignment and phylogenetic analysis

The sequences were assembled, edited, and aligned using BioEdit 7.0.9 [14]. The genomic sequences of the reference vaccine strains used in this study were obtained from the WHO Influenza Sequences Database (http://www.flu.lanl.gov). Phylogenetic analysis of HA1 was performed using a region of 827 bp corresponding to amino acids 21–295 (covering 84% of the HA1 subunit) for H3N2 and 829 bp corresponding to amino acids 17–292 (covering 85% of the HA1 subunit) for H1N1 viruses. Phylogenetic trees were constructed by neighbor-joining method bootstrap analysis ($n = 1{,}000$) using the MEGA (version 4.0) program [21]. For clarity, H3 numbering (based on A/Aichi/2/68 [42]) of the amino acid positions of the HA1 subunit was used for both H3 and H1.

Selection pressure and evolutionary rate analyses

Selection analysis was carried out using probabilistic models of codon substitution implemented in the CodeML program included in the PAML package, version 4.0 [44]. This program employs likelihood models that account for heterogeneous substitution rate ratios (ω = non-synonymous/synonymous substitution or dN/dS) among sites. In this study, we implemented models M0, M1a, M2a, M3, M7, and M8, which were previously tested for their robustness in testing for positive selection [45, 46]. An $\omega > 1$ is considered an indication of positive selection, whereas an $\omega < 1$ implies absence of positively selected sites. Three comparisons were conducted. The first was the neutral model (M1a), which assumes a proportion p_0 of conserved sites with $0 < \omega_0 < 1$ and a proportion $p_1 = 1-p_0$ of neutral sites with $\omega_1 = 1$, against the selection model (M2a), allowing for a third class of sites with $\omega_2 > 1$ (positively selected sites) and a proportion $p_2 = 1-p_0-p_1$. Model M0, assuming a single ω for all sites was compared with M3, which uses an unconstrained discrete distribution with three site classes (ω_0, ω_1, ω_2 with proportions p_0, p_1, p_2, respectively). The third test compared M7 (β model), which uses β distribution with parameters p and q to account for variable ω_0 in the interval of zero to unity against M8, which adds to the β model an extra class of sites with $\omega > 1$ (with p_2 and ω_2). The Bayes approach was then used to calculate the posterior probability that each site identified to be under positive selection ($\omega > 1$) belonged to this class of sites [45]. The likelihood ratio test (LRT) was used to determine whether models (M2a, M3, and M8) allowing for positive selection sites ($\omega > 1$) provide a significantly better fit to the data than the alternative models that do not allow for positive selection or null models (M0, M2a, and M7). When two models are nested, the LRT compares twice the likelihood difference ($2\Delta l$) with a X^2 distribution with the degrees of freedom (df) equal to the difference in the number of free parameters between the two models (check ref. [40, 42, 43] for detailed explanation of the models and their parameters). LRT was performed using the PAML program [44, 45]. Estimates of evolutionary rate (nucleotide substitution rate) were obtained by the maximum-likelihood method using a single dated tips model that assumes a constant rate of substitution [26]. This model was implemented in the BASEML program included in the PAML package, version 4.0 [44].

N-Glycosylation analysis

Potential *N*-glycosylation sites (amino acids Asn-X-Ser/Thr, where X is not Pro) were predicted using the NetNGlyc server 1.0 (available on the www.cbs.dtu.dk website).

Nucleotide accession numbers

The nucleotide sequences used in this study are available in the GenBank database under accession numbers AB271703, AB271704, and AB438225 to AB438363.

Results

Influenza virus A subtypes and amantadine-resistance rates

A total of 1,609 influenza virus isolates were obtained during the eight influenza seasons from 2000 to 2007 (Table 1). Of these, influenza A accounted for 65% ($n = 1041$) and 35% ($n = 568$) were influenza B. Of the influenza A virus isolates, 766 (75.4%) were H3N2 and 275 (24.6%) were H1N1. H3N2 viruses were detected in all seasons, while H1N1 viruses were isolated during five of the eight seasons (no H1N1 viruses were detected in the 2002/2003, 2003/2004, and 2004/2005 seasons). The average age among patients from whom the virus could be isolated was 6.6 ± 4.5 years old, compared to 5.2 ± 4.6 years old for patients for whom no virus was isolated (data obtained from 2001/2002 to 2006/2007 seasons). The vaccination rate among the patients included in the study was 5, 22, 39, 40, 48, and 49%, for the 2001/2002, 2002/2003, 2003/2004, 2004/2005, 2005/2006, and 2006/2007 seasons, respectively.

Amantadine-resistance rates among H3N2 viruses were between 0.7 and 3.4% during 1999/2000–2004/2005 and surged to 100% in 2005/2006 and 79.4% in 2006/2007. In the case of H1N1, amantadine-resistant viruses were not detected until the 2006/2007 season, when 48.2% of the isolates were found to be resistant (Table 1). The M2 gene sequence analysis revealed that all amantadine-resistant H3N2 and H1N1 viruses had a single amino acid change from Ser to Asn at residue 31 (S31 N) in the M2 protein.

Sequence and phylogenetic analysis of the HA1 subunit of the HA gene

A total of 88 H3N2 HA1 sequences (including amantadine-sensitive and resistant viruses), and 56 H1N1 HA1 sequences (including amantadine-sensitive and resistant viruses) in addition to reference sequences of vaccine strains for each subtype were employed in the phylogenetic analysis.

For H3N2, the phylogenetic tree was highly branched, but evolved sequentially in a single linear trunk (Fig. 1a). The tree was interrupted only once in the 2001/2002 season with abortion of the A/Panama/2007/1999 lineage, and the sequences of the 2002/2003 season evolved from those of

Table 1 Incidence of influenza A subtypes and amantadine resistant isolates during the study period

Season	H3N2		H1N1	
	No. of isolates	Amantadine-resistance (%)	No. of isolates	Amantadine resistance (%)
1999–2000	59	2 (3.4)	90	0 (0)
2000–2001	12	0 (0)	15	0 (0)
2001–2002	44	1 (2.3)	59	0 (0)
2002–2003	178	2 (1.1)	0	0
2003–2004	167	2 (1.2)	0	0
2004–2005	142	1 (0.7)	0	0
2005–2006	101	101 (100)	55	0 (0)
2006–2007	63	50 (79.4)	56	27 (48.2)
Total	766	159	275	27

2000/2001, characterized by retaining the signature amino acid substitutions found in the 2000/2001 season strains (e.g. I144N) and loss of those of the 2001/2002 season strains (A106V, N144D, S186V), with the appearance of A/Fujian/411/2002-like strains (Fig. 1a). In the 2006/2007 season, the trunk split into two branches: one contained amantadine-sensitive viruses, and the other accommodated the resistant ones. In total, amino acid mutations at 62 sites in the HA1 subunit were detected for the whole study period (Fig. 1b). Of these, 21 amino acid changes were retained for two or more successive years (Fig. 1c), and 14 of them belonged to the receptor-binding domain (RBD; 190 helix: residues 192 and 193; 220 loop: residues 222, 225, 226, and 227) and/or one of the reported antigenic sites (antigenic site A: residues 131, 144, and 145; B: residues 155, 156, 189, 192, and 193; D: residue 202; and E: residue 83) in the globular head [7, 39, 41].

In the case of H1N1, a multi-furcated tree was formed (Fig. 2a). The tree in the A/New Caledonia/20/1999-like lineage was interrupted in the 2001/2002 season, after which no H1N1 viruses were detected for three consecutive seasons, and the tree evolved again in the 2005/2006 season from the sequences of the 1999/2000 season. However, the trunk was again interrupted and eventually evolved into three major branches in the 2005/2006 and the 2006/2007 seasons. One of the branches included A/Solomon Islands/3/2006, which had quit circulating during the 2006/2007 season, and one of the two branches in the 2006/2007 season (harboring amantadine-sensitive viruses) contained A/Brisbane/59/2007, the recommended vaccine strain for the southern hemisphere in 2008. In total, amino acid changes at 36 sites in the HA1 subunit of H1N1 were detected over the study period (Fig. 2b). Only 11 of these were retained for ≥2 consecutive years (Fig. 2c), of which four amino acid changes occurred in the RBD (190 helix: residue 190) or one of the antigenic sites (site Sa: residue 124; and site Ca1: residues 169 and 270) located in the globular head [7, 41, 47].

Positive selection and evolutionary rate analyses

At the amino acid level, the average *dN/dS* (avg. ω) for the HA1 subunit of H3N2 viruses ranged from 0.41 to 0.44 in all codon substitution models (Table 2). Thus, a non-synonymous substitution has about 41–44% as much chance as synonymous mutations of being fixed. On the other hand, for the H1N1 viruses, the average *dN/dS* ratio (avg. ω) ranged between 0.25 and 0.29, implying that a non-synonymous substitution has about 25–29% as much chance as a synonymous mutation of becoming fixed (Table 2). For HA1 of both H3N2 and H1N1, the selection M2a and M8 models successfully detected positively selected sites ($\omega_2 = 1.48$ with $p_2 = 0.22$, and $\omega_2 = 1.52$ with $p_2 = 0.2$, respectively, for H3N2, and $\omega_2 = 7.16$ with $p_2 = 0.005$, and $\omega_2 = 7.1$ with $p_2 = 0.005$, respectively, for H1N1), but did not significantly provide a better fit to the data when compared with the alternative neutral M1a and beta distribution M7 models, respectively, as determined by the LRT (Table 2). On the other hand, the discrete model (M3) provided a significantly better fit to the data in comparison with the one ratio model (M0); the LRT test statistic for this comparison was $2\Delta l = 2 \times [-2273.09 - (-2285.04)] = 23.9$, with P value < 0.001 with $df = 2$, for H3N2, and $2\Delta l = 27$, with P value < 0.001 with $df = 2$, for H1N1 (Table 2).

In the case of H3N2, the M3 model suggested that approximately 20% ($p_2 = 0.2$) of the sites were under positive selection ($\omega_2 = 1.5$). At a threshold of 95%, only five amino acid sites, namely residues: 50, 131 (antigenic site A), 144 (antigenic site A), 145 (antigenic site A), and 220 (antigenic site B), were under positive selection. In the case of H1N1, the M3 model estimated that ~15% ($p_1 = 0.15$) of the sites were under weak diversifying selection, with $\omega_1 = 1.13$, and only ~0.005% ($p_2 = 0.0049$) of sites were under high positive selection, with $\omega_2 = 7.26$. At 95% threshold, residues: 144, 149, 163 (antigenic site Sa), and 190 (RBD) were positively selected.

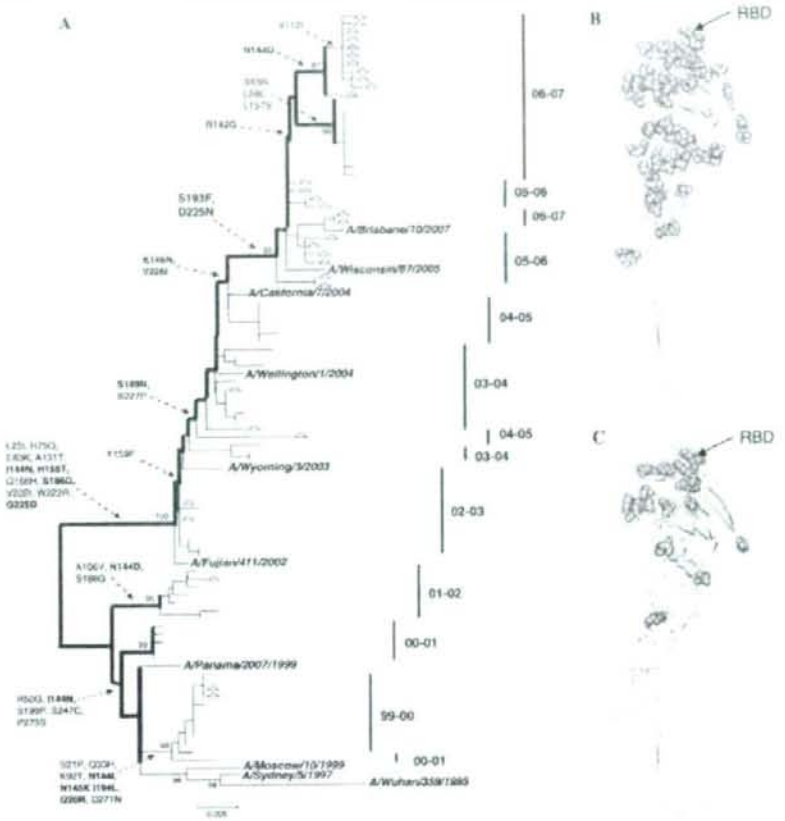

Fig. 1 Sequence analysis of the HA1 subunit of the hemagglutinin (HA) glycoprotein of the human influenza A/H3N2 viruses isolated in Niigata, Japan, during 1999–2007. **a** Phylogenetic tree analysis of the HA1 fragment from strains isolated in Niigata, rooted at A/Wuhan/359/1995. Reference vaccine strains are denoted in italics. A *delta* sign indicates amantadine-resistant strains. Characteristic amino acid mutations are shown on the trunk of the tree (mutations occurring within antigenic sites or RBD are indicated in *bold*). Bootstrap values greater than 70% are also shown. **b** Structure of the HA1 subunit of human H3 (PDB: 2hmg; A/Aichi/1968). The surface-filling models represent all sites at which amino acid substitutions were observed for the whole study period among Niigata isolates. The *grey shading* indicates the RBD. **c** The structure of the HA1 subunit showing sites at which mutations were retained for two or more years. All protein structure figures were generated using the Pymol program [9]

The rate of substitution in the HA1 subunit was 4.58×10^{-3} nucleotide substitutions/site per year (95% confidence interval [CI], 3.9×10^{-3}–5.26×10^{-3}) for H3N2 and 3.83×10^{-3} nucleotide substitutions/site per year (95% CI 3.35×10^{-3}–4.3×10^{-3}) for H1N1 as estimated by the tip date analysis. Furthermore, the ratio of transitions to transversions (ts/tv) for H3N2 was almost half that of H1N1, 3.79–3.86 versus 6.96–7.35, respectively.

N-Glycosylation sites

Nine putative *N*-glycosylation sites (residues: 22, 38, 63, 122, 126, 133, 165, 246, and 285) were identified in the HA1 subunit of the H3N2 isolates (Table 3). These sites were found to be conserved among all isolates obtained in the eight seasons of the study. An additional site at position 144, due to an I144N substitution within antigenic site A

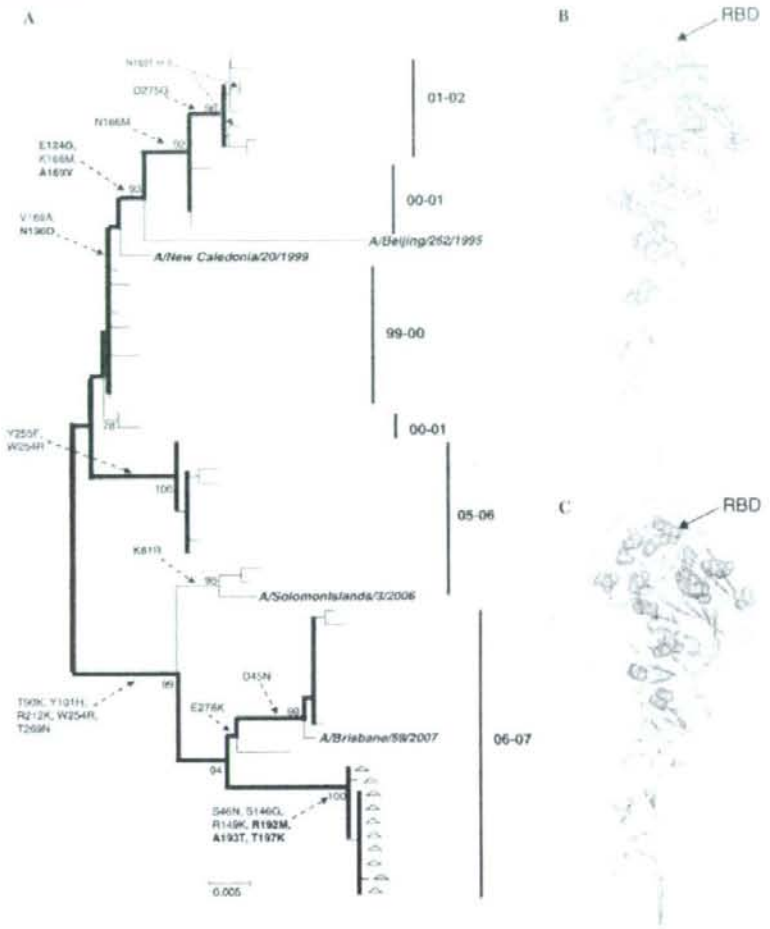

Fig. 2 Sequence analysis of the HA1 subunit of the hemagglutinin (HA) glycoprotein of the human influenza A/H1N1 viruses isolated in Niigata, Japan, during 1999–2007. **a** Phylogenetic tree analysis of the HA1 fragment from strains isolated in Niigata, rooted at A/Beijing/262/1995. Reference vaccine strains are denoted in italics. A *delta* sign indicates amantadine-resistant strains. Characteristic amino acid mutations are shown on the trunk of the tree (mutations occurring within antigenic sites or RBD are indicated in *bold*). Bootstrap values greater than 70% are also shown. **b** Structure of the HA1 subunit of human H1 (PDB: 1ruz; human 1918 HA expressed using a synthetically made gene). The surface-filling models represent all sites at which amino acid substitutions were observed for the whole study period among Niigata isolates. The *grey shading* indicates the RBD. **c** The structure of the HA1 subunit showing sites at which mutations were retained for two or more years. All protein structure figures were generated using the Pymol program [9]

[39], was acquired by the strains collected in the 2000/2001 season. This *N*-glycosylation site was temporarily lost in the 2001/2002 season, due to an N144D substitution, but was then retained in the following seasons until the 2006/2007 season, when only the amantadine-resistant viruses lost it again. Another *N*-glycosylation site (amino acid

Table 2 Log likelihood values and parameter estimates for the selection analysis of the HA1 subunit of the HA genes of human influenza A

Subtype	Model	p^a	l^b	*P* value for LRT	Estimates of parameters[c]	Avg ω	ts/tv
H3N2	M0 (one ratio)	1	−2285.04	<0.0001	$\omega = 0.4327$	0.4327	3.85955
	M3 (discrete)	5	−2273.09		$p_0 = 0.27100$, $p_1 = 0.52312(p_2 = 0.20588)$, $\omega_0 = 0.01554$, $\omega_1 = 0.22964$, $\omega_2 = 1.52649$	0.4388	3.84203
	M1a (neutral)	2	−2273.60	0.6	$p_0 = 0.61684(p_1 = 0.38316)$, $\omega_0 = 0.04099(\omega_1 = 1)$	0.4084	3.79393
	M2a (Positive selection)	4	−2273.09		$p_0 = 0.77498$, $p_1 = 0.0(p_2 = 0.22502)$, $\omega_0 = 0.13644(\omega_1 = 1)$, $\omega_2 = 1.48011$	0.4388	3.84204
	M7 (beta)	2	−2273.65	0.57	$p = 0.01570$, $q = 0.02025$	0.4176	3.81628
	M8 (beta and ω)	4	−2273.09		$p_0 = 0.79370$, $p = 1.47871$, $q = 7.83366(p_1 = 0.20630)$ $\omega_2 = 1.52369$	0.4386	3.84305
H1N1	M0 (one ratio)	1	−1891.75	<0.0001	$\omega = 0.29098$	0.29098	7.10722
	M3 (discrete)	5	−1878.27		$p_0 = 0.84459$, $p_1 = 0.15045(p_1 = 0.00496)$, $\omega_0 = 0.09738$, $\omega_1 = 1.12535$, $\omega_2 = 7.25778$	0.2876	7.36490
	M1a (neutral)	2	−1880.85	0.07	$p_0 = 0.79775(p_1 = 0.20225)$, $\omega_0 = 0.06380(\omega_1 = 1)$	0.2531	6.96159
	M2a (positive selection)	4	−1878.29		$p_0 = 0.81250$, $p_1 = 0.18240(p_2 = 0.0051)$, $\omega_0 = 0.08221(\omega_1 = 1)$, $\omega_2 = 7.16266$	0.2857	7.35049
	M7 (beta)	2	−1881.19	0.06	$p = 0.05925$, $q = 0.16792$	0.2608	7.01889
	M8 (beta and ω)	4	−1878.41		$p_0 = 0.99477$, $p = 0.13758$, $q = 0.41013(p_1 = 0.00523)$ $\omega_2 = 7.09862$	0.2871	7.35652

LRT log likelihood ratio test for each comparison

[a] The number of free parameters in each model

[b] Log likelihood

[c] The parameters in parantheses are not free parameters

position 45) was exclusively found in amantadine-sensitive isolates in the 2006/2007 season. In total, four new predicted *N*-glycosylation sites (45, 122, 133, and 144) that were not present in A/Wuhan/359/1995, the vaccine strain for the 1996/1997 season, were found until the 2006/2007 season (Fig. 1).

In the case of H1N1, seven potential *N*-glycosylation sites (residues: 20, 21, 33, 63, 94, 130, and 163) were conserved throughout the study period except in the 2001/2002 season, when some of the circulating strains lost glycosylation at position 163 within antigenic site Sa (Table 3) [7].

Discussion

During the eight influenza epidemics from 2000 to 2007, H3N2 viruses were the dominant influenza A subtype in five of the eight seasons. The prevalence of amantadine-resistance among H3N2 viruses increased dramatically from 0.7 to 4.3% during the first six seasons to 100% in the 2005/2006 season, in association with dual mutations in the HA1 protein at residues 193 and 225 within the RBD (named clade N) [30, 32]. This high prevalence of resistant viruses was also found in other Asian countries, USA, and Canada [10, 22]. However, viruses sensitive to amantadine fell within the clade N in the HA1 phylogeny in the 2006/2007 season, suggesting a possibility of reverting back from resistance to sensitivity due to continuing viral evolution or competitive disadvantages of resistant viruses against sensitive ones. Amantadine-resistant H1N1 viruses also emerged in the 2006/2007 season, and their HA1 commonly possessed three amino acid substitutions (R192M, A193T, A197K) that were located in the 190 loop of the RBD [47] as in our previous report [32].

The surge in the prevalence of resistant viruses in Japan occurred despite the decrease in amantadine usage from 2.7 million treatment courses in the 2002/2003 season to only 0.1 million in the 2005/2006 season [31], supporting the notion that drug selection pressure was not the sole cause of this sharp rise in resistance rate, but some advantageous mutations located elsewhere in the viral genome might have contributed [33]. As a case in point, the increased prevalence of amantadine-resistant strains was associated with a common amino acid substitution at residue 193 within the RBD for both H3N2 and H1N1. An amino acid mutation at this position was also found in clade I A/H5N1 viruses, which were also resistant to amantadine [38]. This suggests a specific contribution by the substitution at this residue to the wide spread of resistance.

Phylogenetic analysis of the HA1 fragments for both H3N2 and H1N1 subtypes showed that the major cluster of

Table 3 *N*-Glycosylation sites predicted in the HA1 protein of influenza A isolates

Subtype	Season	Amino acid position[a]
H3N2	1999/2000	22,38,63, **122, 126, 133**,165,246, 285
	2000/2001	22,38,63, **122, 126, 133, 144**,165,246, 285
	2001/2002	22,38,63, **122, 126, 133**,165,246, 285
	2002/2003	22,38,63, **122, 126, 133, 144**,165,246, 285
	2003/2004	22,38,63, **122, 126, 133, 144**,165,246, 285
	2004/2005	22,38,63, **122, 126, 133, 144**,165,246, 285
	2005/2006	22,38,63, **122, 126, 133, 144**,165,246, 285
	2006/2007	*S*[b]; 22,38,45,63, **122, 126, 133, 144**,165,246, 285
		R[b]; 22,38,63, **122, 126, 133**, 165,246, 285
H1N1	1999/2000	20,21,33,63,94,130, **163**
	2000/2001	20,21,33,63,94,130, **163**
	2001/2002	20,21,33,63,94,130, **163**[c]
	2002/2003	NA[d]
	2003/2004	NA
	2004/2005	NA
	2005/2006	20,21,33,63,94,130, **163**
	2006/2007	20,21,33,63,94,130, **163**

[a] Bold numbers represent an antigenic binding site

[b] *S* indicates amantadine-sensitive strains and *R* denotes resistant ones

[c] some of the strains lost *N*-glycosylation at this position (3/8)

[d] NA, not applicable; no H1N1 viruses were isolated in these seasons

any given season did not harbor any sequences from the previous season. Moreover, during the study period, influenza viruses were detected only between December and May in Niigata, showing a seasonal pattern. These observations suggested that viruses circulating in each season in Niigata were derived from newly imported strains (external introduction) rather than continuing circulation of the endemic strains from the previous seasons. This seems to be a common feature of seasonal epidemics in temperate-climate areas [25, 27, 29]. On the other hand, the case is different in countries where influenza circulates all year round, such as in Southeast Asia. We previously showed that local epidemic strains in Vietnam from a certain season clustered phylogenetically with some strains from the previous epidemics, providing evidence for local persistence of influenza strains in tropical regions [22].

Amino acid changes in the HA1 subunit of both H3 and H1 were stochastic and scattered all over the protein, and approximately only one-third of these mutations were retained for two or more successive seasons. As most of the retained mutations were confined to the globular part of the HA1 domain, improvement of fitness and evasion of neutralization by the host antibodies were expected [20]. This supports the theory of positive Darwinian selection proposed for influenza A viruses [5, 12]. The total number of mutations in the HA1 of H3 was greater than that of H1, which suggests that a higher selective pressure is being imposed on H3 [43], and this explains the necessity for more frequent updates in the vaccine strains for the H3N2 subtype. Moreover, the characteristic linear trunk in the H3 tree in comparison with the multi-furcated one in H1 could be attributed to a higher tendency of mutations occurring in the latter to revert to an earlier ancestor.

An important point to be noted is that the relatively small number of samples included in our analysis might have biased the shape of the trees for both subtypes. However, repeating the phylogenetic analysis with a larger number of Japanese strains, from this study and from the database, led to more branching or building up on some of the branches, but the tree retained its general structure (data not shown). This could be due to the fact that the main trunk of the tree usually depicts the pathway of advantageous mutations that were fixed by natural selection in both subtypes [24], and thus adding more sequences might capture more variants within each season but would not affect the general number of retained (advantageous) mutations.

The average non-synonymous-to-synonymous substitution ratios (d_N/d_S) for the HA1 of H3N2 and H1N1 viruses in this study did not surpass 1 under any of the models allowing for positive selection. Hence, the HA1 subunit is generally under purifying selection, which lowers the frequency of mutations that impose a negative effect on the fitness of the virus, and only certain sites are affected by adaptive selection. The model M3 (discrete) provided the best fit to the data and suggested five sites (50, 131, 144, 145, and 220) in the HA1 subunit of H3N2 under positive selection at a 95% level. Only residue 145 was previously reported to be positively selected [4], and all of the other identified sites were novel and, except site 50, belonged to the antigenic sites of H3.

In the case of H1N1, we found four novel residues (144, 149, 163, and 190) in the HA1 subunit being positively selected, and two of them (residues 163 and 190) belonged to antigenic sites [7]. A previous study of H1N1 isolates from New York and New Zealand between 1994 and 2005 found no evidence of positively selected residues in the HA1 of H1N1 [43]. These results suggested that positively selected sites might change over different periods of time or in different host populations. Notably, positively selected amino acid position 144 was common to both H3 and H1, though this residue was reported to be within an antigenic site only in H3. The detection of positively selected sites that did not belong to already known antigenic sites [7, 39] raises the need for future studies to elucidate a possible antigenic role for these sites.

Remarkably, positively selected residues, 144 in H3 and 163 in H1, were subject to a gain or loss of *N*-glycosylation

during the study period. An important function of *N*-linked glycosylation of influenza virus proteins is to evade detection by the immune system. The loss or gain of *N*-glycosylation sites is an important mechanism underlying antigenic drift through masking or unmasking of the antigenic sites [1, 36]. Our analysis of the HA1 protein of the H3N2 viruses in this study predicted 9–11 potential *N*-glycosylation sites. In the 2000/2001 season, H3N2 viruses that closely clustered with A/Panama/2007/99 acquired an additional *N*-glycosylation site at the HA1 amino acid 144 (T144N) within antigenic site A. However, this site was lost in the following season due to a point mutation (N144D), which resulted in the discontinuity of this lineage and re-emergence of strains with 144N in the 2002/2003 season. This glycosylation site was further retained until the 2006/2007 season, when the dominantly circulating adamantine-resistant viruses lost it again. Smith et al. [34] reported that a single substitution at amino acid position 145 (N145K) caused a significant antigenic cluster transition. The addition of an oligosaccharide chain to the neighboring site (residue 144) is also expected to have a large impact on antigenicity. These findings suggest an important antigenic role for this site, which was also found to be under selective pressure, and which the H3N2 viruses seemed to use to escape detection by the immune system. Notably, an additional *N*-glycosylation site (S45N) was exclusively detected in all the amantadine-sensitive H3N2 strains isolated in the 2006/2007 season. This site has not yet been reported to possess any antigenic role, and it is therefore unclear, whether it could provide any fitness advantage for the amantadine-sensitive lineage.

In the case of H1N1, the seven predicted *N*-glycosylation sites were retained in all strains except in the 2001/2002 season, when some circulating viruses lost one of these sites due to an amino acid replacement N163T or S, antigenic site Sa. This lineage failed to prevail in the following season, and later viruses again retained *N*-glycosylation on this site. These data highlight the importance of *N*-glycosylation in the evolution of influenza A viruses, and especially those occurring within antigenic or positively selected sites should be considered when choosing vaccine strains.

Furthermore, to gain better insight into the evolutionary patterns observed in the HA1 phylogenies of influenza H3N2 and H1N1, we calculated their nucleotide substitution rates in this study. The evolution rate of the H3N2 viruses was found to be slightly faster than that of the H1N1 viruses (4.58×10^{-3} vs. 3.83×10^{-3} nucleotide substitutions/site per year). This difference was intensified by the higher fixation rate of non-synonymous mutations (dN/dS) of HA1 in H3N2 than in H1N1 (0.439 vs. 0.286, respectively), explaining the faster drift in H3N2 viruses. To test whether our results are solely an artifact due to the use of more H3N2 sequences in the analysis, we repeated our test with a smaller number of H3N2 sequences ($n = 60$), and again, a higher rate of substitution for H3N2 was obtained (data not shown). Thus, influenza A/H1N1, as an older subtype, evolves more slowly than the relatively newly emergent A/H3N2, probably due to better adaptation of the former to the human host [24]. This could also be seen in the higher number of mutations observed in the HA1 of H3. The higher variation of HA genes in H3N2 viruses could explain the dominant circulation of these viruses in this study as well as in other reports [11]. Nevertheless, dominant circulation might in turn impose more selective pressure on H3N2 viruses to mutate.

In general, our estimated evolution rates for both H3N2 and H1N1 fell within the range of RNA viruses including influenza [13, 16, 19], although it was more than twice as high as that reported recently for a global collection of H3N2 viruses during 1997–2005 [48]. To account for any differences in that period or in methodologies, we analyzed the evolution rate for a global collection of viruses obtained from the Influenza Virus Resource database (http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html) from a period corresponding to our study. Again, the global evolution rates were lower in comparison with those in our study (data not shown). This supports the notion that viral phylogenies constructed from biased sampling of global isolates might mask the complex dynamics underlying influenza virus evolution within discrete populations, maybe because most influenza virus sequencing done as a part of surveillance programs is focused on detection of serologically novel strains [25]. Future studies of evolution rates of viruses obtained in one fixed area, ideally the tropics, where epidemics occur throughout the year, are warranted for better understanding of evolutionary dynamics of influenza.

The study of the phylodynamics of influenza viruses and its underlying mechanisms is fundamental for understanding how these viruses evolve in response to host immunity and vaccination [24]. In conclusion, we demonstrated that faster evolution and larger diversity of H3N2 viruses in comparison with that of H1N1 is mainly attributable to a higher fixation rate of non-synonymous mutations in the former. We show, for the first time, evidence of the presence of positively selected sites in H1N1 (sites 144, 149, 163, and 190). We also report four novel sites in H3N2 (sites 50, 131, 144, and 220) being under selective pressure. *N*-glycosylation at positively selected sites (e.g. residue 144 in the H3) seemed to contribute to improved viral immune evasion. At a threshold of less than 90%, more sites were found to be under selective pressure (data not shown). These sites may change to strongly selected positions over time through continuous evolution of influenza virus due to host immunological pressure. Therefore, future monitoring of changes in these

sites, especially those that could lead to loss or gain of *N*-glycosylation, might provide potentially important information on which variants might dominate in future epidemics.

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