Reassortment between human A(H3N2) viruses is an important evolutionary mechanism

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Abstract

Phylogenetic relationships of whole genomes of H3N2 viruses circulating in Germany during a 6-year period from 1998 to 2005 revealed the co-circulation of different lineages of viruses. Multiple reassortment events occurred during this time between viruses belonging to different lineages or different subgroups. Strains isolated during 1998–1999 were characterised by a surprisingly high heterogeneity and multiple reassortment events. Seventy percent of the examined 1998–1999 viruses had completely different genome compositions. To our knowledge, such an exceptional high proportion of different reassortant strains, encompassing all eight genome segments, have not been described before. In contrast, only one reassortant virus was prevalent during 1999–2000 even though two of the three 1998–1999 lineages were co-circulating. Reassortant viruses were isolated also in each of the other seasons. However, the proportion of H3N2 viruses with different genome compositions varied from season to season. Strains with a reassortant NA played an important role and were also detected during 2003–2004 and 2004–2005 accounting for 45% and 70% of the circulating H3N2 viruses, respectively. Moreover, different reassortment events occurring during these seasons included also the PB1, PB2 and NP genes. The results presented here emphasize that genetic reassortment is an important factor in the evolution of H3N2 viruses and highlight the need for a comprehensive analysis of influenza viruses, especially with regard to the annual vaccine composition.

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Keywords: Influenza A(H3N2) virus; Whole genome analysis; Reassortment, Evolution

1. Introduction

One of the characteristic features of influenza viruses is their segmented genome, which allows for the exchange of the eight gene segments between virus strains. Genetic reassortment between different co-infecting influenza A virus subtypes from various species may create new human subtypes with drastic antigenic changes referred to as antigenic shift. Reassortment between human and avian subtypes of influenza A viruses was the reason for the occurrence and spread of the H2N2 virus causing the “Asian flu” pandemic in 1957 and the H3N2 strain causing the “Hong Kong Flu” pandemic in 1968, respectively [1,2]. Multiple reassortment events have been described among late H2N2 and H3N2 viruses suggesting that H2N2 viruses continued to circulate after 1968 and that establishment of H3N2 viruses in humans was associated with multiple reassortment events [3]. A reassortant H1N1 virus with the polymerase and NP genes derived from a H3N2 virus circulated in several countries from 1978 to 1980 [4,5]. H1N2 viruses which contained seven genes of an H3N2 precursor were isolated sporadically in China between December 1988 and March 1989 but did not persist or appear to spread beyond China [6]. An H1N2 virus appeared 13 years later and was associated with outbreaks in Egypt, Israel and the UK. Subtype H1N2 viruses circulated also in other parts of the world causing sporadic infections [7] but did not become established in the human population. These H1N2 viruses arose by genetic reassortment between recently cocirculating human H1N1 and H3N2 viruses, between 1999 and 2001 [8].

Even if pandemic influenza occurs occasionally, interpandemic influenza remains the major cause of morbidity and mortality. It is estimated that during the last century annual epidemics have had an even greater cumulative impact than the three pandemics [9]. The two surface glycoproteins...
hemagglutinin (HA) and neuraminidase (NA) comprise the principal immunizing antigens of the influenza virus. The HA and NA genes mutate with high frequency [10,11] resulting in the accumulation of point mutations that may lead to gradual antigenic change of the surface glycoproteins known as antigenic drift. The severity of influenza seasons differs from year to year and is mainly associated with the emergence of immunologically distinct strains known as drift variants. Every few years influenza epidemics boost the mortality rate causing thousands of additional deaths in countries such as the United States or Germany [12–14].

The analyses of the HA and NA of seasonal circulating strains demonstrate the ongoing antigenic and genetic drift of influenza viruses. The timely detection of new drift variants is a prerequisite for an optimal vaccine composition. Understanding the evolution of influenza viruses and prediction of new emerging strains is very important for the selection of vaccine strains. There are some reports indicating that not only antigenic drift but also genetic reassortment may contribute to the variability and evolution of A(H3N2) viruses. Reassortment of NA genes was demonstrated between two lineages of A(H3N2) viruses, A/Beijing/353/89 and A/Beijing/32/92, during their circulation [15]. Limited gene reassortment for some of the internal genes was shown for the first time by a Japanese group [16]. Reassortment events detected for the external and internal genes of H3N2 virus isolated in Germany during 1998–1999 prompted us to examine the origin of all eight genes of H3N2 viruses circulating during distinct seasons to get more information about the appearance of intra-subtype reassortment and its contribution to the evolution of influenza A(H3N2) viruses.

2. Material and methods

Influenza A(H3N2) viruses isolated in Germany from October 1998 to April 2005 were obtained from the strain collection of the National Influenza Centre. Between 10 and 15 strains of each season were selected to represent different regions of the country. Influenza viruses isolated in Germany are named according to the county where the sample was taken. The abbreviations used for different counties are as follows: Bayern (BAY), Baden-Württemberg (BWB), Berlin (BLN), Brandenburg (BBG), Bremen (BRE), Hamburg (HAM), Hessen (HES), Mecklenburg-Vorpommern (MVP), Nordrhein-Westfalen (NRW), Niedersachsen (NSA), Rheinland-Pfalz, Saarland (SAL), Sachsen (SAS), Sachsen-Anhalt (SAT), Schleswig-Holstein (SHO), and Thüringen (THR). Recent drift variants were included as reference strains in this study which were, with the exception of A/Christchurch/28/03, recommended as vaccine strains. Viruses circulating from 1998 to 2003 are represented by the reference strains A/Sydney/5/97 (Sydney/97), A/Moscow/10/99 (Moscow/99) and A/Panama/2007/99 (Panama/99). H3N2 viruses isolated during the last three seasons are represented by the strains A/Wyoming/03/03 (Wyoming/03), A/Christchurch/28/03 (Christchurch/03), A/Wellington/01/04 (Wellington/04) and A/California/07/04 (California/04). These strains were obtained from Dr. Alan Hay (WHO Collaborative Centre, London).

For RT-PCR, viral RNA was extracted from 200 µl cell culture supernatant using the RTP DNA/RNA Virus Kit (InViTek, Berlin, Germany). The cDNA synthesis was carried out using a 5 µl aliquot of RNA and amplification of influenza virus specific sequences was performed using the high fidelity Phusion DNA polymerase (Finnzymes, Finland). The amplified fragments covered the following genome regions (from nucleotide position): PB1 22–1388, PB2 165–1071, PA 81–948, hemagglutinin (HA, HA1 domain) 50–1050, nucleoprotein 20–1530, neuraminidase (NA) 07–1430, matrix 17–980 and non-structural (NS) 13–843 (primer sequences available on request). The ampli-cons were purified using the QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and directly sequenced using the ABI Prism Dye terminator III cycle sequencing kit (Applied Biosystems, Germany) on a DNA automated sequencer (Applied Biosystems, Foster City, California, USA). Phylogenetic trees were constructed using a DNA distance matrix, the neighbour-joining method and bootstrap analysis [17] and TreeView [18]. The nucleotide sequence data determined in this study can be retrieved from the Influenza Sequence Database, Los Alamos (accession numbers ISDN183807–ISDN183809, ISDN183812, ISDN186517–ISDN186600, ISDN187149–ISDN187217, ISDN187227, ISDN187228, ISDN187233–ISDN187249, ISDN188294–ISDN188352, and ISDN188677).

3. Results


Phylogenetic analysis of the external genes of viruses from the season 1998–1999 revealed a high degree of variability. The HA genes of eight isolates were closely related to that of the reference strain Moscow/99 (clade B) whereas five strains had HA genes which were closer to that of the reference strain Panama/99 (Fig. 1 clade C). The NA genes of 1998–1999 viruses fell broadly into three groups. Two viruses grouped with the reference strain Moscow/99 (clade B) whereas one of them was closer to that of H3N2 viruses circulating in later seasons. Five strains belonged to the Sydney/97 clade (clade A). A third group of viruses had NA genes that were close to that of the Panama/99 strain (clade C). Comparable results were also found for the internal genes in general. The polymerase genes (for PB1 see Fig. 1) as well as the NP gene (Fig. 1), the M, and NS genes of 1998–1999 viruses belonged either to the Moscow/99, the Panama/99 or to the Sydney/97 group (clade B, C or A, respectively). A comparison of the eight genes of the examined 1998–1999 strains revealed a surprisingly high heterogeneity in the composition of gene...
segments that belonged in variable proportions to the Sydney/97, Moscow/99, and Panama/99 lineages (Table 1). Each of the strains listed in this table is characterised by a different genome composition. Seventy percent of the strains analysed from this season were reassortants with different genome compositions indicating that multiple reassortment events occurred during this time. The remaining 30% had genome compositions that were already identified among the 70% group.

3.2. Season 1999–2000

The external as well as the internal genes of 14 H3N2 viruses from 1999–2000 clustered either with those of the strains Moscow/10/99 or Panama/99 (see Fig. 1 for selected

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A reference strain to that an individual gene segment is closely related is abbreviated as follows: A/Sydney/5/97, S; A/Moscow/10/99, M; A/Panama/2007/99, P; A/New York/82/01, NY; I, interim between Sydney/97 and Moscow/99.

Fig. 1. Phylogenetic analysis of the HA1 domain of the HA genes (HA), the NA genes (NA), the PB1 genes (PB1), and the NP genes (NP) of influenza viruses circulating in Germany from 1998 to 2005. Reference strains A/Sydney/5/97, A/Moscow/10/99, A/Panama/2007/99, A/New York/82/01, A/Christchurch/28/03, A/Wellington/01/04 and A/California/07/04 are shown in bold. Influenza viruses isolated in Germany and the reference strains with the exception of A/New York/82/01 were sequenced as described in Section 2. Sequence data of A/New York/82/01 were obtained from the Influenza Sequence Database (Los Alamos, USA). Bootstrap values are shown for main clades labelled as A–G. The horizontal bar indicates the percentage difference of the sequences based on nucleotides.
strains, clades B and C). All of these strains were homogeneous regarding the polymerase, the HA, NA and the M genes. Interestingly, no strain with a Panama/99-like NA and only one strain with a Panama/99-like NS gene was detected during 1999–2000 whereas such genes were prevalent the season before. Contrary to this, only Panama/99-like M genes were identified during 1999–2000 whereas Moscow/99-like genes were prevalent during 1998–1999. Comparative analysis of the genes of all viruses analysed so far revealed that all strains were reassortants. The vast majority (90%) of viruses had the same composition possessing Panama/99-like PB2, HA and M genes and Moscow/99-like PB1, PA, NP and NS genes, respectively.


The season 2002–2003 was characterised by the appearance of the first A/Fujian/411/02-like (Fujian/02) viruses. However, the vast majority (85%) of the H3N2 viruses identified in Germany was antigenically and genetically A/New York/55/01-like. Therefore, only such New York/55/01-like viruses from 2002–2003 were included in this study. The external genes of A(H3N2) viruses circulating during 2001–2002 and 2002–2003 clustered within clade D that is represented by the reference strain A/New York/55/01 and the strain A/New York/82/01 (Fig. 1). The internal genes of H3N2 viruses from the 2002–2003 and the previous season were genetically closely related to the A/New York/82/01 which was, therefore, selected as a reference strain for viruses circulating during these both seasons (PB1 and NP genes shown in Fig. 1 clusters D-1 and D-2). Two subgroups among the 2002–2003 viruses were identified with each containing about 30% of the viruses analysed. Two viruses were identified in subgroup B, possessing either a reassortant PB1 or a M gene, respectively. Viruses circulating between 2001 and 2003 were much more homogeneous than those circulating between 1998 and 2000. Comparative analysis of the genes of all viruses revealed only limited reassortment between H3N2
viruses circulating during 2001–2002 and 2002–2003. However, HA and NA analysis of 13 Fujian/02-like strains from 2002–2003 not included in this study revealed that about 70% of them were reassortants containing a New York/01-like NA gene.

3.4. Season 2003–2004

All of the H3N2 viruses identified during 2003–2004 were antigenically close to Wyoming/03/, a representative of the new drift variant Fujian/02. Phylogenetic analysis revealed that not only the external, but also all the internal genes of 15 viruses from 2003–2004 belonged to two distinct clades or lineages (HA, NA, PB1 and NP genes shown in Fig. 1). One HA lineage is represented by the reference strain Christchurch/03 (clade E) and the other one by the reference strain Wellington/04 (clade F). All Christchurch/03-like viruses were reassortants. The NA and the internal genes evolved from those of New York/01-like viruses (clade D-3). The HA gene, however, was derived from a precursor not belonging to the New York/01 clade (Fig. 1). About 47% of the strains were identified as Wellington-like and 53% as Christchurch-like, respectively. Three other reassortment events were detected among the analysed strains. The strain A/HES/16/04 had a Wellington/04-like HA, but the remaining genes were Christchurch/03-like. The strain A/NRW/31/04 possessed a Christchurch/03-like HA and seven Wellington/04-like genes (Fig. 1). The third reassortant viruses 1887/04 was Wellington/04-like with the exception of the PB2 gene that were closely related to that of the new variant California/04 and PB2 genes of H3N2 viruses from 2004–2005 (not shown here).
3.5. Season 2004–2005

Phylogenetic relationships of the HA gene showed that all H3N2 viruses analysed so far were genetically closely related to California/04 (Fig. 1 (clade G)). The HA genes of these California/04-like viruses evolved from those of Wellington/04-like viruses circulating during 2003–2004. Three of the ten strains analysed from 2004–2005 possessed HA and NA genes that were closely related to those of California/04. Seven strains from 2004–2005 contained NA genes that clustered together with NA genes of H3N2 viruses isolated during the previous season (Fig. 1, clade F). These viruses built a separate cluster as supported by a bootstrap value of 98. Since all of the H3N2 viruses possessed California/04-like HA genes and 70% of them Wellington/04-like NA genes, at least 70% of the strains analysed herein were reassortants. Analysis of the PB2, PA and the NP genes of H3N2 viruses from 2004–2005 revealed that these genes were closer related to those of the California/04 than to any other reference strain (see Fig. 1 for PB1, PB2 and PA not shown). The M and the NS genes of California/04 and Wellington/04 were very similar and belonged to the same group which was strongly supported by low bootstrap values (not shown). Among the California/04-Wellington/04 reassortants another reassortment event was identified. One of these viruses had a California/04-like PB1 gene whereas six viruses contained a PB1 gene that was derived from a Wellington-like precursor (Fig. 1). Therefore, phylogenetic analysis of the whole genome revealed that three groups of viruses with distinct genome composition co-circulated during 2004–2005.

4. Discussion

Recurrent epidemics of influenza are caused by the frequent emergence of new antigenic variants. With co-circulation of two influenza A virus subtypes and two antigenically distinct lineages of B viruses, genetic reassortment
has an important role in antigenic drift [19,20]. Genetic reassortment of the NA gene between two variants of H3N2 viruses was first described in 1996 [15]. Evolutionary analysis of the NS gene led to the conclusion that reassortment between co-circulating viruses is a rare event [21,22]. Phylogenetic relationships of all eight RNA segments of 10 H3N2 viruses isolated from 1993 to 1997 were analyzed to define the evolutionary pathways of all genes. It was shown that the lack of correlation between the topologies of the phylogenetic trees of the genes encoding the external and internal proteins was a reflection of genetic reassortment [16].

Phylogenetic relationships determined in this study covered the external as well as the internal genes of about 85 H3N2 viruses circulating in Germany from 1998 to 2005. In Germany, the influenza season 1998–1999 was characterized by a co-circulation of Sydney/97-, Moscow/99- and Panama/99-like viruses. The reference strains Sydney/97 and Moscow/99 were antigenically more different than the strains Moscow/99 and Panama/99 that represent two different genetic clades (lineages) although they are antigenically very similar. Comparative analyses of all the genes of H3N2 viruses analysed so far have shown an extraordinary high level of reassortment among the viruses of 1998–1999 of which about 70% had distinct genome compositions. To our knowledge, such a high number of reassortants with complete different genome compositions has been never described before. Moscow/99- and Panama/99-like viruses circulated also during the season 1999–2000 but only a few reassortants with different genome compositions were detected. The vast majority of the viruses (90%) were reassortants with the same genome composition possessing Moscow/99-like PB1, PA, NA, and M genes whereas the PB2, HA, and M genes were closely related to Panama/99. We do not have an explanation for the high number of reassortants during 1998–1999 and the prevalence of only one reassortant strain during 1999–2000, but we assume that the H3N2 viruses of 1999–2000 might have achieved the best evolutionary fitness.

A reassortant H3N2 virus having an HA that was antigenically and genetically similar to the New York State viruses possessing Moscow/99-like PB1, PA, NA, and M genes whereas the PB2, HA, and M genes were closely related to Panama/99. We do not have an explanation for the high number of reassortants during 1998–1999 and the prevalence of only one reassortant strain during 1999–2000, but we assume that the H3N2 viruses of 1999–2000 might have achieved the best evolutionary fitness.

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H3N2 viruses circulating in Germany, the USA [26], Australia and New Zealand [23].

Phylogenetic relationships performed in this study have shown a divergent evolution of the HA and NA genes of H3N2 viruses circulating recently in Germany. The three polymerase, the NP and NS genes of viruses circulating from 1998 to 2004 evolved along two distinct pathways whereas the pathways of the M genes differed from those of the other genes. These findings are in accordance with previous studies describing a divergent evolution of HA and NA genes of H3N2 viruses isolated in Canada between 1997 and 2000 [27]. Moreover, phylogenies of the whole genomes revealed that the evolutionary pathways of the external genes were not linked to those of the internal genes as it was recently shown for viruses circulating in Japan between 1994 and 1997 [16].

In summary, phylogenetic studies of whole genomes of H3N2 viruses circulating in Germany from 1998 to 2005 revealed the co-circulation of different lineages. Multiple reassortment events occurred during this time between viruses belonging to distinct lineages or distinct subgroups. The exceptional high level of reassortment encompassing all eight genome segments detected during the season 1998–1999 resulted in a high percentage of viruses with distinct genome compositions. Reassortant strains were isolated also in each of the other seasons. However, the proportion of H3N2 viruses with different genome compositions varied from season to season. Strains with a reassortant NA were after 1999–2000 mainly detected during 2003–2004 and 2004–2005 accounting for 45% and 70% of the circulating H3N2 viruses, respectively. The results presented here emphasize that genetic reassortment is an important factor in the evolution of H3N2 viruses and highlight the need for a comprehensive analysis of influenza viruses, especially with regard to the annual vaccine composition.

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