Antigenic and genomic characterization of human influenza A and B viruses circulating in Argentina after the introduction of influenza A(H1N1)pdm09

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This study was conducted as part of the Argentinean Influenza and other Respiratory Viruses Surveillance Network, in the context of the Global Influenza Surveillance carried out by the World Health Organization (WHO). The objective was to study the activity and the antigenic and genomic characteristics of circulating viruses for three consecutive seasons (2010, 2011 and 2012) in order to investigate the emergence of influenza viral variants. During the study period, influenza virus circulation was detected from January to December. Influenza A and B, and all current subtypes of human influenza viruses, were present each year. Throughout the 2010 post-pandemic season, influenza A(H1N1)pdm09, unexpectedly, almost disappeared. The haemagglutinin (HA) of the A(H1N1)pdm09 viruses studied were segregated in a different genetic group to those identified during the 2009 pandemic, although they were still antigenically closely related to the vaccine strain A/California/07/2009. Influenza A(H3N2) viruses were the predominant strains circulating during the 2011 season, accounting for nearly 76% of influenza viruses identified. That year, all HA sequences of the A(H3N2) viruses tested fell into the A/Victoria/208/2009 genetic clade, but remained antigenically related to A/Perth/16/2009 (reference vaccine recommended for this three-year period). A(H3N2) viruses isolated in 2012 were antigenically closely related to A/Victoria/361/2011, recommended by the WHO as the H3 component for the 2013 Southern Hemisphere formulation. B viruses belonging to the B/Victoria lineage circulated in 2010. A mixed circulation of viral variants of both B/Victoria and B/Yamagata lineages was detected in 2012, with the former being predominant. A(H1N1)pdm09 viruses remained antigenically closely related to the vaccine virus A/California/7/2009; A(H3N2) viruses continually evolved into new antigenic clusters and both B lineages, B/Victoria/2/87-like and B/Yamagata/16/88-like viruses, were observed during the study period. The virological surveillance showed that the majority of the circulating strains during the study period were antigenically related to the corresponding Southern Hemisphere vaccine strains except for the 2012 A(H3N2) viruses.

INTRODUCTION

In April 2009, a novel reassortant H1N1 influenza virus carrying genes from North American and Eurasian swine influenza viruses spread among people in Mexico. This A(H1N1)pdm09 virus contained a combination of gene segments that had never been reported in swine or human influenza viruses in the USA or elsewhere. The NA and M gene segments have their origin in the Eurasian swine...
genetic lineage. Viruses with NA and M gene segments in this lineage derived from a wholly avian influenza virus and were thought to have entered the Eurasian swine population in 1979. They continued to circulate throughout Eurasia, and were not previously reported outside Eurasia (Garten et al., 2009). The HA (haemagglutinin), NP and NS gene segments are in the classical swine North American lineage. PB2 and PA derived from the avian North American lineage and PB1 from the human seasonal H3N2 virus. This new influenza virus spread quickly across the USA and Canada, and subsequently around the world [Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009]. Argentina was one of the first countries to experience the A(H1N1)pdm09 virus transmission coincident with the usual seasonal influenza, currently running from May to September (Estenssoro et al., 2010; Libster et al., 2010). The first confirmed case of influenza A(H1N1)pdm09 was identified in Argentina on 28 April 2009. The virus did not spread until mid-May when the first group affected was school-aged children in Buenos Aires, the capital city. A shift in the groups most affected with severe cases was observed when the new virus widely circulated in the community (Van Kerkhove et al., 2011; Azziz-Baumgartner et al., 2012). Three-quarters of confirmed influenza A(H1N1)pdm09 fatalities occurred among persons aged <50 years. However, the highest incidence of H1N1pdm09 fatalities occurred among persons aged 50–65 years and children aged <5 years (half of them <1 year). Persons with co-morbidities, pregnant women and those who received antiviral drugs late were over-represented among influenza A(H1N1)pdm09 deaths in Argentina, though the timeliness of antiviral treatment improved during the pandemic (Balanzat et al., 2012).

Argentina has a country-wide surveillance system that collects virological and epidemiological data. Antigenic and genomic analyses of clinical isolates provide information about the evolution and geographical spread of new emerging epidemic strains and ultimately help identify suitable virus strains to be included in the trivalent influenza vaccine (Cox et al., 1994). Argentina has an annual vaccination programme comprising all at-risk groups, achieving high levels of coverage especially during 2010 (AMH, 2011).

The aims of this study were to have a picture of the influenza seasonality after the introduction of the new A(H1N1)pdm09 strain, and to achieve the antigenic and genomic characterization of influenza A(H3N2), A(H1N1)pdm09 and B strains circulating in Argentina in the period 2010–2012. The relationship of the circulating strains and the corresponding vaccine strains recommended was analysed for this period.

**METHODS**

**Specimen collection.** Influenza virological surveillance is conducted routinely by the National Influenza Centre (NIC) located at the Instituto Nacional de Enfermedades Infecciosas (INEI), ANLIS ‘Carlos G. Malbrán’. The virological surveillance system is based on the year-round sampling effort coordinated by the NIC and 65 laboratories that together comprise the National Influenza and other Respiratory Viruses Laboratory Network (RVLN), as part of the influenza surveillance system of the Argentinean Ministry of Health (AMH). Nasopharyngeal aspirates from paediatric outpatients and inpatients, and throat or nasal swabs from adult outpatients and inpatients with acute respiratory infections are collected and examined by immunofluorescence assay for diagnosis of influenza A, influenza B and other respiratory viruses, including respiratory syncytial virus, adenovirus, parainfluenza viruses and metapneumovirus. The detection of type A and B influenza viruses is also performed by a one-step real-time reverse transcriptase PCR (rt RT-PCR) assay (WHO, 2009), in line with the recommendations of the AMH (AMH, 2010b, 2012a, 2013a). The RVLN analysed 71 277 acute respiratory infection samples in 2010, 67 174 in 2011 and 69 000 in 2012 for diagnostic testing. Clinical specimens positive for influenza A or B are routinely submitted to the NIC by laboratories of the RVLN for further characterization. Therefore, 1147 clinical samples positive for influenza were submitted to the NIC in 2010, 1118 in 2011 and 2212 in 2012. Neither written informed consents nor explicit ethical approval were sought as this study was only observational and carried out as part of a routine virological surveillance (anonymously, without identification of patients), as stated in the Terms of Reference for NICs by the World Health Organization (WHO).

**Influenza-like illness (ILI) notification.** These data were generated by the AMH based on data collected from hospital notifications using the following ILI definition: abrupt onset of fever (>38 °C), one or more respiratory symptoms (non-productive cough, sore throat, rhinitis) and one or more systemic symptoms (myalgia, headache and/or severe malaise) (AMH, 2013a).

**Subtyping, virus isolation and antigenic characterization.** The subtyping of influenza A viruses was carried out by rt RT-PCR assay using specific primer/probe sets for the H1 and H3 HA s of current viruses using the Centers for Disease Control and Prevention (CDC) influenza subtyping kit. A subset of positive influenza A(H3N2), A(H1N1)pdm09 and B clinical samples (612 in 2010, 563 in 2011 and 824 in 2012) was selected for isolation in mammalian cells, taking into account the date of sampling, the specimen preservation conditions and the geographical location, in order to be able to obtain isolates with different characteristics from different regions of the country throughout the study period. Parental Madin–Darby canine kidney (MDCK) and MDCK-SIAT1 cells engineered to express increased levels of α2,6-sialyltransferase (Matrosovich et al., 2003) were used in order to isolate viruses from clinical specimens. MDCK-SIAT1 was used for the isolation of influenza A(H3N2) viruses, and MDCK for influenza A(H1N1)pdm09 and influenza B viruses. The presence of virus in the culture was confirmed by direct immunofluorescence and/or haemagglutination assays following standard protocols using suspensions of guinea pig (0.75%, v/v) red blood cells performed no later than 7 days post-inoculation (Barr et al., 2010). A subset of virus isolates was antigenically characterized by haemagglutination inhibition (HI) test using post-infection ferret antisera raised against the following egg- or cell-grown reference and vaccine influenza A and B viruses: A/California/7/09(H1N1)pdm09 (2010–2012 vaccine strain), A/Perth/16/2009(H3N2) (2010–2012 vaccine strain), A/Brisbane/10/2007(H3N2) (2008–2009 vaccine strain), A/Victoria/361/2011(H3N2) (2013 vaccine strain), B/Brisbane/60/08 Victoria lineage (2010–2012 vaccine strain), B/Malaysia/2506/04 Victoria lineage (2006–2007 vaccine strain) and B/Wisconsin/1/10 Yamagata lineage (2013 vaccine strain). During the 2011 and 2012 influenza season, HA and HI tests were performed for influenza A(H3N2) viruses in the presence of 20 nM oseltamivir to circumvent the neuraminidase-mediated binding of these viruses to the red blood cells (Lin et al., 2010). Part of these data were complemented by two WHO Collaborating Centres for the Reference and Research on Influenza located at the...
CDC, Atlanta, GA, USA, and the National Institute for Medical Research, London, UK.

**Gene sequencing and analysis.** Of the 461 influenza A(H3N2), 242 influenza A(H1N1)pdm09 and 318 influenza B tissue culture isolates obtained between 2010 and 2012, a subset of 132 isolates was selected for phylogenetic analysis based on the HA partial sequences: 80 influenza A(H3N2), 35 influenza A(H1N1)pdm09 and 17 influenza B viruses. Strains selected for this study represent the viral types and subtypes circulating in each particular season in different regions of the country. RNA was extracted from 140 μl of influenza A and B isolates using the QIAmp viral RNA mini kit (Qiagen), according to the manufacturer’s instructions. Reverse transcriptase PCR was performed with a One-Step RT-PCR kit (Qiagen) using specific primers to amplify the HA1 region of the HA: influenza A(H3N2), 986 bp; influenza A(H1N1)pdm09, 981 bp; and influenza B, 1039 bp. PCR products were purified with the Qiaquick PCR purification kit (Qiagen) and sequenced to examine mutations at specific positions. Templates were labelled by cycle sequencing reactions with fluorescent dye terminators (Big Dye terminator v3.1 cycle sequencing kit; Applied Biosystems) and products were analysed using an ABI 3100 Avant Genetic Analyzer (Applied Biosystems). Sequences were analysed using the BioEdit program, version 7.0.5.3 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html); MEGA 4 (Tamura et al., 2007) and MEGA 5 (Tamura et al., 2011) programs were used to reconstruct the phylogenetic trees using the neighbour-joining distance method. Tree topology was supported by bootstrap analysis with 1000 replicates.

**RESULTS**

**Seasonal surveillance**

The different types and subtypes of influenza viruses received by the WHO NIC and the incidence curve of ILI cases in Argentina in 2010–2012 are shown in Fig. 1. During 2010, the ILI historical peak was not observed at the beginning of the winter season [June–July, epidemiological week (EW) 23–30] based on the total number of cases in the country, as expected in relation to the endemic channel. Otherwise, an increase in the number of ILI notifications was observed, exceeding the alert zone, in August (EW 31–35) and between October and December (EW 40–51) according to the circulation of influenza B and A(H3N2) viruses, respectively (AMH, 2010a). The predominant circulating virus was influenza B (615 positive samples out of a total of 1147 positive samples received at the NIC, 54 %). Within type A, the H3 subtype (n=506, 44 %) predominated over A(H1N1)pdm09 viruses; unexpectedly, after the 2009 pandemic, only a few influenza A(H1N1)pdm09 (n=26, 2 %) viruses were confirmed in the laboratory.

In the following season, an increase in ILI notifications, exceeding the alert zone of the endemic corridor, was observed between March and April (EW 11–15). However, the major circulating influenza was detected between July and September (EW 27–39) (AMH, 2012b). The detection of influenza A viruses began in May (EW 18–19), with a peak in July–August (EW 27–35), which declined by the end of October (EW 43–44). During the same year, the activity of influenza was mainly associated with influenza A(H3N2). The number of specimens positive for influenza received at the NIC were 844 A(H3N2) (75.5 %), followed by 268 A(H1N1)pdm09 (24 %) and a low number (n=6) of influenza B cases (0.5 %).

![Fig. 1. Weekly ILI cases reported in Argentina and influenza-positive samples received at the NIC during the 2010–2012 seasons.](image-url)
In 2012, ILI notifications remained within the expected range into the safety zone for the first weeks of the year until the end of August (EW 36). After that, and until mid-October (EW 42), ILI notifications exceeded the expected values, within the alert zone, in line with A(H1N1)pdm09, A(H3N2) and B co-circulation (AMH, 2012c). This season started late, in comparison with the previous two seasons, with an increase in viral detections from mid-June (EW 24). This influenza activity was reflected in the number of positive samples that the NIC received: 869 influenza B (39.3 %) and 677 influenza A(H3N2) viruses (30.6 %), with a peak in mid-September (EW 38), after the circulation of influenza A(H1N1)pdm09 with a peak in August (EW 32–35).

The number of influenza-positive specimens received at the NIC, and the number of strains recovered from cell culture by type and subtype are shown in Table 1. The mean rate of virus isolation in tissue culture in 2010–2012 was between 46–56 %. Due to the large area of Argentina, approximately 2 791 810 km², samples have to be transported over long distances to reach the NIC, which is a disadvantage in the isolation performance. Clinical specimens collected promptly and of good quality (ideally within 3 days after the onset of clinical symptoms), among others, will improve the recovery rate in tissue culture.

### Antigenic and genetic characteristics of the viruses

A spatial–temporal representative number of influenza-positive specimens collected during the three seasons analysed were antigenically and genetically characterized. Table 2 reports the number of influenza A(H3N2), A(H1N1)pdm09 and B viruses included in the antigenic analysis for each season, performed by HI test.

#### Influenza A(H1N1) viruses

Since 2009, all seasonal influenza A(H1N1) viruses from Argentina were replaced by A(H1N1)pdm09 viruses. HI tests using post-infection ferret antisera [1 influenza isolate A(H1N1)pdm09 in 2010, 16 isolates in 2011 and 36 in 2012] indicated that the majority of A(H1N1)pdm09 virus isolates from Argentina during the study period remained antigenically homogeneous and closely related to the vaccine virus A/California/7/2009.

Phylogenetic trees show amino acid residues that define the genetic groups most predominant over recent years. Also, sporadic observations of particular amino acid substitutions or polymorphisms can be observed in these trees. Currently, the phylogenetic tree of the A(H1N1)pdm09 HA1 gene can be divided into eight major genetic subgroups. Subgroup 5 viruses were found worldwide with signature amino acid substitutions of D97N, R205K, I216V and V249L. Subgroup 6 viruses, which were also circulating worldwide, carry amino acid substitutions D97N and S185T. Subgroup 7 viruses, mainly seen in Europe, Asia and Oceania, carry the amino acid substitution A197T, and many also carry amino acid substitutions S143G and S185T (WHO, 2012a). Sequence analysis of the HA1 region of A(H1N1)pdm09 viruses (Fig. 2) indicated that Argentinean viruses fell into at least three genetic subgroups (5, 6 and 7).

<table>
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<tr>
<th>Year</th>
<th>A(H3N2)</th>
<th>A(H1N1)pdm09</th>
<th>B</th>
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<td>26</td>
<td>615</td>
<td>1147</td>
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<tr>
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<td>268</td>
<td>6</td>
<td>1118</td>
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<tr>
<td>Total</td>
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<td>960</td>
<td>1490</td>
<td>4477</td>
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<td>8</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>110</td>
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<td>1</td>
<td>8</td>
<td>48</td>
<td>110</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 1. Virological surveillance, 2010–2012 seasons influenza-positive clinical specimens received at the NIC and virus isolates

Table 2. Isolates characterized by HI test with ferret antisera
that were antigenically indistinguishable. The majority of the viruses circulating in Argentina between 2011 and 2012 belonged to subgroups 6 (29/35, 83 %) and 7 (4/35, 11 %). Due to the low circulation of A(H1N1)pdm09 during 2010, it was not possible to sequence and therefore make a phylogenetic study of these strains.

**Influenza A(H3N2) viruses**

Regarding antigenic analysis, HI assays were performed to test 8 strain isolates in 2010, 68 in 2011 and 8 in 2012. Isolates recovered in 2010 and the majority of the 2011 circulating viruses were antigenically closely related to the A/Perth/16/2009 vaccine virus, except for one strain from 2011, which was similar to the previous vaccine virus A/Brussels/10/07. However, the 2012 virus isolates were antigenically closely related to A/Victoria/361/2011, recommended by the WHO as the H3 component for the 2012–2013 Northern Hemisphere (WHO, 2012c) and 2013 Southern Hemisphere formulations (WHO, 2012d). As observed worldwide, the majority of A(H3N2) isolates genetically analysed in this report belong to the A/Victoria/208/2009 (78/80, 97.5 %) genetic clade (subgroups 3C, 4, 5 and 6). According to the WHO classification, the majority of the HA1 A(H3N2) sequences analysed in Argentina fell into group 6 [24/31 (77 %) in 2010; 30/35 (86 %) in 2011] and group 5 [7/12 (58 %) in 2012] (Fig. 3). Isolates included in the smaller A/Perth/16/2009 genetic clade (reference vaccine strain for the 2010, 2011 and 2012 season) carry signature amino acid substitutions E62K (in epitope E) and N144K (in epitope A). Within the A/Perth/16/2009 group, there are subgroups 1 and 2 with several amino acid substitutions. Within the A/Victoria/208/2009 clade, the most common strains circulating in Argentina belonged to subgroups 5 and 6. Subgroup 5 has signature amino acid substitutions D53N, Y94H, I230V and E280A in the HA1. Subgroup 6 isolates carry additional amino acid substitutions, S199A and I230V. Of note, several substitutions accumulated were exposed in the HA and could have a significant impact on the antigenicity of the virus.

**Influenza B viruses**

Regarding the virological characteristics of the B virus isolates from Argentina in 2010, all the 110 influenza B viruses tested belonged to B/Victoria lineage. The 2011 season was characterized by a widespread activity of A/H3N2 viruses (75.5 %), which prevailed over A(H1N1)pdm09 strains (24 %), and only a few influenza B cases were identified by the RVLN (0.5 %). None of these influenza B viruses were recovered from tissue culture. In 2012, influenza B viruses belonging to both B/Victoria/2/87 and B/Yamagata/16/88 lineages co-circulated, and 56 isolates were studied by HI test. Most of the strains that belonged to Victoria lineage (86 %) were antigenically closely related to the B/Brisbane/60/2008 vaccine strain. Meanwhile, Yamagata lineage viruses were antigenically closely related to the B/Wisconsin/1/10 strain. The 23 viruses genetically characterized in 2012 fell into clade 1A of the Victoria lineage and presented substitutions A169B, A202V, K307R and V146I (Fig. 4). Three influenza B Yamagata lineage viruses fell into clade 2, represented by the reference strain B/Brisbane/3/2007 (Fig. 5), and had substitutions R48K, P108A, T181A and D196N. The remaining HA1 sequences fell into clade 3, represented by the reference strain B/Wisconsin/1/2010, and carried substitutions S150I, N165Y, G229D and D196N.

**DISCUSSION**

Surveillance of influenza viruses is essential for updating vaccines, for tracking the emergence of drug-resistant viruses and for monitoring emerging variants. It also gives important insights into the mechanisms of virus evolution, such as the correlation between antigenic differences and changes in the sialic acid receptor-binding properties of the HA glycoprotein (Lin et al., 2012). This study aimed to reveal the viral activity and the antigenic and genomic features of a set of representative influenza circulating virus in Argentina over the period 2010–2012.

Because Argentina is a Southern Hemisphere country with temperate climate extending from latitudes 21° to 55° 03′, influenza seasons occur usually from May to September, as a major winter peak. Occasionally, influenza outbreaks have been observed outside the expected seasonality, mainly in cities located in extreme latitudes (Savy et al., 2007).

Before the influenza pandemic, 800 000 and 200 000 influenza vaccine doses were used in 2007 in adults and paediatric individuals from Argentina, respectively. While in 2008, 900 000 and 100 000 doses were available for adults and paediatric patients, respectively. In 2009, the recommended vaccine strains were A/Brisbane/59/2007 (H1N1)-like virus (seasonal), A/Brisbane/10/2007 (H3N2)-like virus and B/Florida/4/2006-like virus. The doses available for adults and paediatric individuals were 1 200 000 and 100 000, respectively. In contrast, in the post-pandemic period 8 473 460, 4 936 090 and 4 442 724 doses were used in Argentina in 2010, 2011 and 2012, respectively (AMH, 2014). During the three post-pandemic seasons, influenza virus circulation was detected from January to December: types A and B, and all the current subtypes of human influenza viruses.
In 2010, a monovalent vaccine influenza A(H1N1)pdm09 was available early in March, followed by a trivalent vaccine administered before the onset of the seasonal influenza. During the 2011 and 2012 seasons, the vaccine formula recommended by the WHO for the Southern Hemisphere included strains A/Perth/16/2009(H3N2), A/California/07/2009(H1N1)pdm09 and B/Brisbane/60/2008 (Victoria lineage). The vaccination coverage in 2010 reached 93.4% (AMH, 2011). Meanwhile, the vaccination coverage in 2011 and 2012 was 88 and 86.9%, respectively (AMH, 2012d, 2013b).

During the three study periods, a delay in the viral circulation trend was observed compared with previous years, especially in 2012 (WHO, 2012b). The increase in the number of ILI cases correlated with the viral circulation in the community.

A combination of antigenic and genetic analyses is used for the identification of potential epidemic-pandemic strains and for consideration of their inclusion in vaccines. Argentina was one of the countries in South America most affected by influenza A/H1N1)pdm09 (Van Kerkhove, 2011).

**Fig. 3.** Phylogenetic analysis of A(H3N2) isolates. The phylogenetic tree was built as described in Fig. 2. Genetic subgroups are labelled 1–2, 3A, 3B, 3C, 4–7. Victoria and Perth clades are distinguishable.

**Fig. 4.** Phylogenetic analysis of B/Victoria lineage isolates. The phylogenetic tree was built as described in Fig. 2. Genetic clades are labelled 1A and 1B.
et al., 2011), with cases registered as early as May 2009 (Giglio et al., 2012). The 2010 influenza season was the first epidemic after the occurrence of the 2009 pandemic. During this season, the circulation of A(H1N1)pdm09 was rare (27 laboratory confirmed cases all through 2010), and mostly detected in May. In general, global outbreaks decreased due to A(H1N1)pdm09 viruses during this period, leading to the declaration of the post-pandemic phase by the WHO on 10 August 2010 (WHO, 2010a).

In 2011, although the world had moved into the post-influenza pandemic period, influenza A(H1N1)pdm09 activity increased in the southern part of South America thus becoming regional – as categorized by the CDC (2013). At least two regions were affected in May–June (EW 18–26) in Argentina, the Dominican Republic, Uruguay and South Africa, and declined in August–September (EW 32–39) (WHO, 2011). In Argentina, between June and July of that season, an increase in influenza A(H1N1)pdm09 cases occurred in Mendoza province focused on at-risk unvaccinated patients, in the context of a mild influenza season in the rest of the country where A/Perth/16/09 (H3N2)-like viruses were circulating predominantly. It should be noted that one strain isolated in 2011 (A/Buenos Aires/656/11) demonstrated more than a fourfold reduction in the HI test titre compared with the homologous titre obtained using post-infection antisera generated against the A/California/07/2009 vaccine strain (data not shown). Since 2009, it has been observed that most of these viruses with reduced HI test titres had amino acid changes in HA positions 153–157 and were generated as a consequence of tissue culture isolation (Klimov et al., 2012).

Fig. 5. Phylogenetic analysis of B/Yamagata lineage isolates. The phylogenetic tree was built as described in Fig. 2. Genetic clades are labelled 2 and 3.


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