MAJOR ARTICLE

Evolutionary dynamics of 2009 Pandemic Influenza A(H1N1) in South Africa from 2009-2010

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Abstract:

Background

2009 Pandemic Influenza A(H1N1) was first detected in June 2009 in South Africa and later resulted in extensive transmission throughout Africa. Established routine surveillance programmes and collaboration between private and public sector laboratories allowed for comprehensive molecular epidemiological and antigenic investigation of the first and second waves of 2009 Pandemic Influenza A(H1N1) in South Africa.

Methods:

We screened 9792 and 6915 specimens from patients with Influenza like illness (ILI) or severe acute respiratory infection (SARI) symptoms from surveillance programmes in hospitalised or outpatients in South Africa in 2009 and 2010, respectively for Influenza by RTPCR. Influenza-positive specimens were subjected to genetic and antigenic characterisation. Bayesian and Maximum likelihood analyses of the Hemaglutinin (HA)genes of 96 2009 Pandemic Influenza A(H1N1) strains were used for molecular epidemiological investigations. Hemaglutination inhibition assays and sequencing of the PB2 and neuraminidase genes were used to investigate pathogenicity and resistance mutations.

Results:

The 2009 Pandemic Influenza A(H1N1) epidemic occurred as a second epidemic peak following seasonal H3N2 cases in 2009 and in 2010. Progressive drift away from the A/California/7/2009 vaccine strain was observed at both the nucleotide and amino acid level with 2010 strains clustering separate to 2009 strains. A few unique clusters of amino acid changes in severe cases were identified, but most strains were antigenically similar to the vaccine strain and no resistance or known pathogenicity mutations were detected.

Conclusion:

Despite limited drift observed over the 2 seasons in South Africa, circulating 2009 Pandemic Influenza A(H1N1) strains remained antigenically similar to strains identified in other Northern and Southern hemisphere countries from 2010 and 2011.

Introduction

Since the emergence of 2009 Pandemic Influenza A(H1N1), laboratories throughout the world have monitored the evolution of the virus, producing essential information for vaccine strain selection and drug resistance [1]. Global circulation of 2009 Pandemic Influenza A(H1N1) viruses is thought to be the result of multiple introductions of distinct lineages rather than a single viral lineage [2]. Fereidouni et al., (2009) showed the existence of 2 distinct clusters of 2009 Pandemic Influenza A(H1N1) virus circulating globally as early as March to September 2009 [3].

Antiviral treatment of cases of 2009 Pandemic influenza A(H1N1) infection was limited to the use of neuraminidase inhibitors (oseltamivir and zanamivir) throughout the pandemic as adamantine resistance mutations in the M2 protein were widespread[4]. Few neuraminidase inhibitor resistant strains have been detected to date, and most were associated with the prophylactic use of oseltamivir or individuals with immune immunosuppression[5-10]. All oseltamivir-resistant viruses contain the H275Y mutation, which renders the virus resistant to oseltamivir but sensitive to zanamivir [11, 12].

Pathogenic markers have not yet been defined although it has been postulated that a mutation observed in the HA1 gene fragment at position D222G, first detected in patients with severe disease in Norway[13, 14] and confirmed in several other regions, may be associated with more severe disease[15, 16]. Potential emergence of viruses with increased pathogenic phenotype through accumulation of mutations on HA calls for molecular epidemiological investigation and surveillance of cases of mild compare to severe disease[17].

The molecular epidemiology of influenza in Africa is poorly defined, and data concerning the evolution of pandemic strains in Africa is not readily available. The high prevalence of HIV infection in Africa may provide opportunities for accumulation of mutations due to prolonged viral shedding and development of resistance[18], which emphasizes the need for investigation of the evolution of strains from this region.

South Africa is the major financial centre of the continent and the hub for travel from Europe, South America, the USA and Asia into most Southern African countries . During the 2010 Influenza season, South Africa hosted one of the largest sporting events in the world, the FIFA Soccer World Cup (11 June-11July), sparking fears that South Africa could become a mixing pot for new strains and start new epidemics spreading to the rest of the world.

Here we describe the Influenza season of 2009-2010 and the emergence and evolution of 2009 Pandemic Influenza A(H1N1) in South Africa from July 2009 through December 2010. Using specimens collected from multiple surveillance systems in South Africa, we examined the geographic and temporal distribution of pandemic strains, evaluated genetic and antigenic drift in HA genes and amino acid changes in cases of mild and severe disease, and investigated pathogenic markers in the HA and PB2 genes and resistance markers in the NA genes.

Materials and Methods

Specimens and viruses:

    Surveillance Programmes in South Africa

Influenza surveillance in South Africa consists of 3 main active surveillance programmes run by the National Influenza Centre (NIC), National Institute for Communicable Diseases (NICD): 1) The passive "Viral Watch Programme" for ILI at 246 sentinel healthcare facilities in all 9 provinces; 2) The passive "Enhanced Viral Watch Programme" – established June, 2009, following the emergence of 2009 Pandemic Influenza A(H1N1), which targets hospitalised patients with SARI in 11 hospitals covering 8 provinces that was established to detect 2009 Pandemic influenza A(H1N1); and 3) The SARI active sentinel surveillance programme, which was established in January 2009 and monitors cases of SARI in hospitalised patients in 4 hospitals in 3 provinces. In all 3 surveillance programs, nasopharyngeal (NP) and/or oropharyngeal (OP) specimens were tested for influenza by real time reverse transcription polymerase chain reaction (RT-PCR).During the pandemic specimens from cases of severe disease or deaths were also confirmed for private and public sector laboratories across the country at the NIC.

For this study, we selected 72/1206 2009 Pandemic Influenza A(H1N1).positive NP and/or OP specimens from the 3 active surveillance programmes (46 ILI and 26 SARI) from the 2009 season and 24 2009 Pandemic Influenza A(H1N1).samples from the 2010 season(17 SARI and 7 ILI). Specimens were selected to represent all geographic regions of the country, from the start to the end of the epidemic in each year.

Influenza diagnoses and typing:

Specimens were tested using the real-time PCR protocol developed by the WHO Collaborating Centre (CC) for Influenza, Centres for Disease Control and Prevention

(CDC), USA for the detection and characterisation of 2009 Pandemic Influenza A(H1N1).virus [19].

Extraction, amplification and sequencing

RTPCR positive clinical specimens were used directly for sequencing or, in cases where the titre was too low, 2 day Mardin Darby Canine Kidney (MDCK) shell vial cultures were used. Total nucleic acid was extracted from specimens using the MagNA Pure 96 DNA and Viral nucleic acid kit on the MagNA Pure 96 instrument (Roche Applied Science, Mannheim Germany) as per manufacturer's instructions. The HA and NA gene segments were amplified, and partial sequencing was performed using an adapted version of the Genome Sequencing protocols of WHO CC for influenza, CDC Atlanta, USA [20] as described below.

HA gene

One step RT-PCR of the complete HA gene was followed by four nested PCR's to amplify four overlapping fragments of the HA gene (Table 1) using the Access RT PCR kit (Promega, Madison, WI USA) according to manufactures instructions. PCR cycles were as follows: 45˚C for 45min, 94˚C for 2min, followed by 35 cycles of (94˚C for 30sec, 48˚C for 1min, 68˚C for 1min) and 68˚C for 7min. The nested PCR's were performed using the Go®Taq Hot Start polymerase (Promega, Madison, WI USA) according to manufactures instructions. and cycled at 94˚C for 2min, and 40 cycles of (94˚C for 30sec, 56˚C for 45sec, 72˚C for 45sec) and 72˚C for 5 min.

NA and PB2 fragments

The smaller fragments containing the H275Y and E627K mutations for the NA and PB2 genes were amplified separately using the Titan One Tube RT PCR system (Roche Applied Science, Manneheim Germany) according to manufacturers' instructions. I Reactions were cycled at 50°C for 30min, 94°C for 2min, followed by 40 cycles of (94°C for 30sec, 50°C for 30sec, 68°C for 1min) and 68°C for 7min.

Amplicons were purified using the ExoSAP-IT enzyme system (USB Corporation, Cleveland OH) and sequenced using the Big Dye terminator V3.1 cycle Sequencing Ready Reaction kit (ABI, Foster City, USA) with M13 sequencing primers. Reactions were purified with the BigDye® XTerminator Purification kit (ABI, Foster City, USA) . and run on the 3130XL genetic analyzer.

Antigenic characterization

Hemagglutination Inhibition Assays (HIA) assays were performed using the WHO influenza reagent kit for the identification of influenza isolates supplied by the WHO CC for Reference and Research, Melbourne, Australia, using turkey red blood cells.

Phylogenetic analysis

A partial region of the HA gene i(nucleic acids 73-1200) was targeted for phylogenetic analysis, while the HA1 region was used for amino acid investigations (amino acid position 8-327). Sequences were aligned with ClustalX version1.83[21]. The evolutionary analyses over time of the 2009 Pandemic Influenza A(H1N1) strains were conducted using a coalescent Bayesian Markov chain Monte Carlo (MCMC) approach as implemented in the BEAST 1.6 package [22] using the HKY substitution and Gamma site Heterogeneity models. A strict molecular clock setting was used to estimate rate of

evolution from data with an exponential growth coalescent prior option. Operators were optimized over several runs before running 10 million MCMC chains and sampling every 5000 trees. Dates were introduced according to the month of isolation (January 2009 was set as month 1 and January 2011 as month 25). Statistical uncertainty in the data was reflected by the 95% highest probability density (HPD) values and given as percentages posterior probability on the branches. Results were examined with TRACER from the BEAST package [22]. Convergence was assessed with Effective Sample Size (ESS values, after burning of 10 million steps). Maximum clade credibility trees were generated using Tree Annotator and a burning of 200 to obtain 10% burning values. FigTree v1.2.2 (http://tree.bio.ed.ac.uk/) was used for the visualization of annotated trees.

Maximum likelihood (ML) and neighbor- joining trees using the Tamura-Nei model were generated with MEGA version 5 [23]. Trees were drawn to scale, with branch lengths measured in number of substitutions per site. Positions containing gaps and missing data were eliminated. Statistical support was provided by bootstrapping over 100 and 1000 replicates, respectively, and values >70 are shown[24]. Pair-wise distance analyses between years were determined as the proportion of differences (P-distance). Estimates of evolution were based on the ratio of non-synonymous substitution per non-synonymous site (Ka) to synonymous substitutions per synonymous site (Ks) calculated with the Modified Nei-Gojobori method in MEGA where ka/ks>1 indicates positive selection using MEGA version 5 [23].

Ethical considerations

The SARI surveillance protocol was reviewed and approved by University of the Witwatersrand Human Research Ethics Committee (protocol number M081042). Molecular epidemiology analyses of ILI cases from the two viral watch programs are covered by ethical clearance for viral surveillance for NICD by the same committee.

RESULTS

The 2009 and 2010 influenza seasons

The NIC received 9792 specimens in 2009 and 6915 in 2010 from patients with ILI or SARI symptoms from all three surveillance programmes (Figure 1). In 2009, 2442/9792(24.9%) and in 2010 1316/6915(19.03%) of cases tested positive for Influenza. In 2009 influenza positive specimens were detected in two waves, the first being H3N2 with 1096(11%) cases (week 18-35), followed by a second wave of 2009 Pandemic influenza A(H1N1) cases (n=1206;12%) from week 25-42. Only 1%(n=140) of specimens tested were influenza B. The 2010 Influenza season consisted mostly of influenza B (n=709; 10% specimens received) detected from week 8-42. Apart from 2 travel-imported 2009 Pandemic influenza A(H1N1) cases in weeks 2 and 14, influenza A cases were only detected in week 23 onwards with H3N2 detected from week 23 in 333 (5%) of specimens and 2009 Pandemic influenza A(H1N1) viruses detected from week 27 onward in 274 (4%) cases. Sporadic 2009 Pandemic influenza A(H1N1) activity was observed until week 52; the later cases were mostly associated with travellers during the South African summer.

Molecular epidemiological analyses of Pandemic Influenza A(H1N1) strains.

Genetic Drift in the HA gene

Bayesian evolutionary analyses of the HA1 region of the HA gene revealed 3 distinct 2009 Pandemic Influenza A(H1N1) genetic lineages circulating in the country during 2009 (Figure 2), which is indicative of multiple introductions to South Africa

during the first wave of the pandemic. Evolution over time is demonstrated from the first appearance of 2009 Pandemic Influenza A(H1N1) in April 2009 in Mexico (indicated in red), with South African strains from 2009 (shown in blue) in the first branch being closest to the Mexico strains isolated from June to April 2009. The next two branches likely originated from a second and third introduction. South African strains in the second branch clustered with isolates from England and New York, USA while the third cluster had mostly 2010 sequences and were close to isolates from Australia and the USA.

Strains from all of the provinces clustered with all three branches, suggesting community spread through the country. Limited drift away from the initial strains suggests positive selection with an estimated clock rate of 0.9×10^4. 2009 strains from South Africa clustered on two separate branches, separate from 2009 isolates. The first smaller group (indicated in purple) appears to include strains that drifted from 2009 strains and were identified in June to September in South Africa. Strains in the bottom branch were the furthest from the root of the tree and consisted of strains identified from August to November 2010. This lowest branch clustered with the consensus sequence generated by the CDC Atlanta Influenza Division for 2011 strains identified in the Northern hemisphere, indicated by (H1N1pdm_HA_ Consensus 2011_270).

Maximum likelihood analyses identified branches distinguished by unique amino acid changes in Figure 3, where cluster 1 was characterised by Q293H, cluster 2 by P83S, S203T, I321V, and cluster 3 by a D222E change. The D222G mutation has been associated with fatal cases in Norway and several other countries *[14]*, but it was not present in South African strains. The D222E mutation was identified in 6 strains in 2009

in South Africa, of which two had mild influenza like illness and four were severely ill with SARI. This change was not observed in strains circulating during 2010. The 2010 strains grouped separately from the 2009 strains and were further from the root of the tree than the 2009 strains. P-distance analysis of 2009 strains indicated 0.3-0.6% difference in nucleotide and 0.7-1% amino acid level relative to the vaccine strain and for 2010, 0.7-1% nucleotide and 1.5-2% amino acid differences.

Three unique lineages were identified in 2010. The first had an amino change from aspartic acid to asparagine at position 97. The second clustered closely with 2009 strains and had four amino acid changes; N31D, S162N, A186T and V272I. The third clusters were closest to a strain from Brisbane in 2010 and had the N125D and V272F mutation. The 2010 strains generally clustered together with the most recent strains identified during the 2010/2011 northern hemisphere influenza season.

South African 2009 strains had an average of 0.5% nucleotide relative to 0.9% amino acid differences to the vaccine strain; in contrast, the 2010 strains had 0.8% nucleotide relative to 1.6% amino acid differences relative to the vaccine, suggesting a progressive although low level accumulation of amino acid changes. Calculations for positive selection indicated Ka/Ks values of 0.6 for South African 2009 strains relative to the vaccine and 0.8 for 2010 but 1.6 for the international 2011 strains which suggest positive selection.

Antigenic analysis of 18 2009 Pandemic Influenza A(H1N1) viruses suggested that most 2010 strains (n= 14) produced HAI results similar to the A/California/7/2009 control, although two medium reactors and two low reactors were identified with

A/California/7/2009-like ferret antiserum. Both 2010 genetic clusters contained viruses which were high reactors to antiserum to the current vaccine strain, A/California/07/2009. Two of four strains that were identified as low reactors by HAI had two amino acid changes at N125D and V272F (Figure 3 and 5) relative to the vaccine strain. However, these viruses did not cluster with the majority of South African 2010 strains.

Comparison of strains from cases of ILI and SARI

Strains from patients with ILI and SARI were compared by phylogenetic clustering (Figure 4) and amino acid analyses (Figure 5) to identify any changes associated with disease severity. No unique clustering of strains from patients with mild or severe disease was observed phylogenetically (Figure 4). A few unique amino acid changes were identified in certain strains (Figure 5) including 2 clusters of mutations at position S185T and Q293H that were present in 4/18 South African strains from patients with SARI but not in ILI cases.

Sequencing of the PB2 gene of 30 strains, targeting the E627K mutation which is a known pathogenic marker *[25]* identified the wild type, E627, mutation in both ILI and SARI cases (results not shown).

Geographic distribution

Phylogenetic comparison of specimens from different geographic regions indicated strains originating in different provinces clustered together and in all 3 branches suggesting a wide distribution of strains across the country (Figure 2).

NA gene antiviral resistance mutations

Of the 118 samples that were tested for Oseltamivir resistance, (n=68 from ILI and n=50 from SARI), all were sensitive to Oseltamivir and none had the resistance mutation *[1]*.

Discussion:

2009 Pandemic influenza A(H1N1) was first identified in South Africa on the 15th of June 2009, in a traveller from North America, in the midst of the annual influenza A season and two months after it first emerged in Mexico*[26]*. Well established routine surveillance programmes and collaboration between private and public sector laboratories helped to detect the first cases. A total of 12640 cases and 93 deaths were recorded nationwide in 2009 by both private and public sector laboratories *[27]*.

In this study we describe for the first time the temporal and molecular evolution of pandemic influenza in South Africa over the first two-years of the pandemic. 2009 Pandemic Influenza A(H1N1) cases occurred in 2009 as a second wave following seasonal H3N2 cases in South Africa. A second 2009 Pandemic Influenza A(H1N1) period occurred in 2010 with less intensity and in concomitance with Influenza B circulation, once again as a second peak following Influenza A H3N2 activity. Despite the fears of the influenza season coinciding with the 2010 FIFA soccer world cup in South Africa, peak influenza A activity occurred in the second week of June for H3N2 and first week of July for 2009 Pandemic influenza A(H1N1) viruses. Both were detected at a much lower level than in 2009 and towards the end of the World Cup. In

contrast to many northern hemisphere countries, 2009 Pandemic influenza A(H1N1) cases did not occur during the South African summers of 2009 or 2010 apart from a few imported cases.

Molecular epidemiological analyses of the South African 2009 Pandemic Influenza A(H1N1) strains suggest multiple introductions in 2009 and at least three clusters in 2010. Genetic drift was visible over time with the 2010 strains clustering separately from 2009 strains and further from the Mexico- and the A/California/7/2009 vaccine strain. 2010 South African strains clustered with the northern hemisphere consensus 2011 strain. Antigenically most strains were still similar to the vaccine although a few low reactors were identified. Mutations at positions N125D and V272F were identified in the low reactors, and these mutations should be monitored in the future. No oseltamivir resistance mutations were identified in either season.

Despite the rapid spread of the 2009 Pandemic Influenza A(H1N1) virus, it remains as pathogenic as many seasonal influenza viruses and although severe in certain adults, children and pregnant women, overall the mortality rate was only about 0.2%-0.04% *[28] [29]*. None of the South African strains had the known PB2 pathogenic markers [25] or the putative HA D222G mutation identified in patients that died in Norway. Mutations at S185T and Q293H in HA were identified in a number of patients with SARI symptoms that should be investigated further to determine any association with more severe disease.

A limitation in the present study was that many of the deaths and cases of severe disease were identified in the private sector and RNA or small amounts of specimens were received by the NIC was often not enough for virus isolation or sequencing. This

prohibited identification of the exact source of the identified clusters or investigation of pathogenic markers in deaths.

Conclusions

Progressive genetic drift away from the original A/California/7/2009 vaccine strain is apparent from estimations of positive selection. A few amino acid changes identified in cases of SARI warrants further investigation as possible pathogenic markers in South Africa. Full genome sequencing as well as investigation of host factors and virus phenotypic characteristics will determine if other factors may be associated with pathogenic differences seen in individuals which are limitations in the current study. Antigenic investigations do however suggest that these strains are still similar enough to A/California/07/2009 to be covered by the current vaccine.

special thanks also to all of the viral watch doctors as well as doctors and medical personnel participating in the enhanced viral watch and SARI surveillance programmes.

and 2010. The D222G mutation identified in Norway is indicated by a grey bar while two clusters of mutations identified in South Africa are indicated in open bars by the arrows in the alignment at S168T and Q294H.

Table 1 : RT PCR and nested PCR primers used for amplification of HA, NA and PB2 fragments

Supplementary table: Accession numbers for South African and global strains used in the phylogenetic analysis of the HAI region of the HA gene

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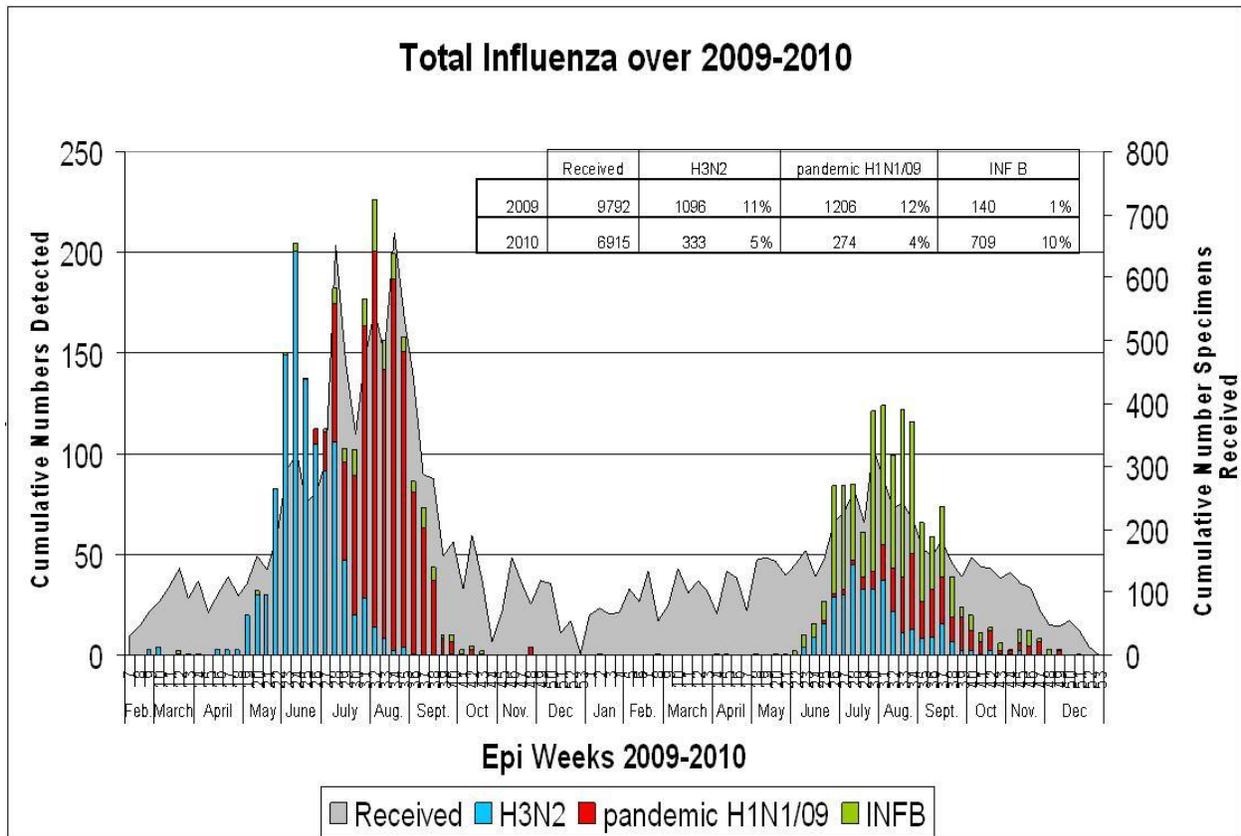
Figure 1: Total number of specimens received and tested by the National Influenza Centre South Africa between 2009 and 2010 and the corresponding subtypes identified for each influenza season.

Figure 2: Bayesian evolutionary analysis over time of 2009 Pandemic Influenza A(H1N1)strains identified in South Africa from 2009 to 2010 compared to global strains during the same time period. The 2009 strains are indicated in blue and the 2010 strains are indicated in purple. The vaccine strain and original Mexican strain is shown in red and the consensus sequence generated for 2011 strains by CDC Atlanta in yellow.

Figure 3: A rooted maximum likelihood tree showing the evolutionary relationship between the 2009 (indicated by triangle) and 2010 South African strains (indicated by square) relative to the current vaccine strain, A/California/07/09. The consensus sequences containing 2011 global strains are indicated by an arrow. Amino acids defining clusters have been annotated on the tree.
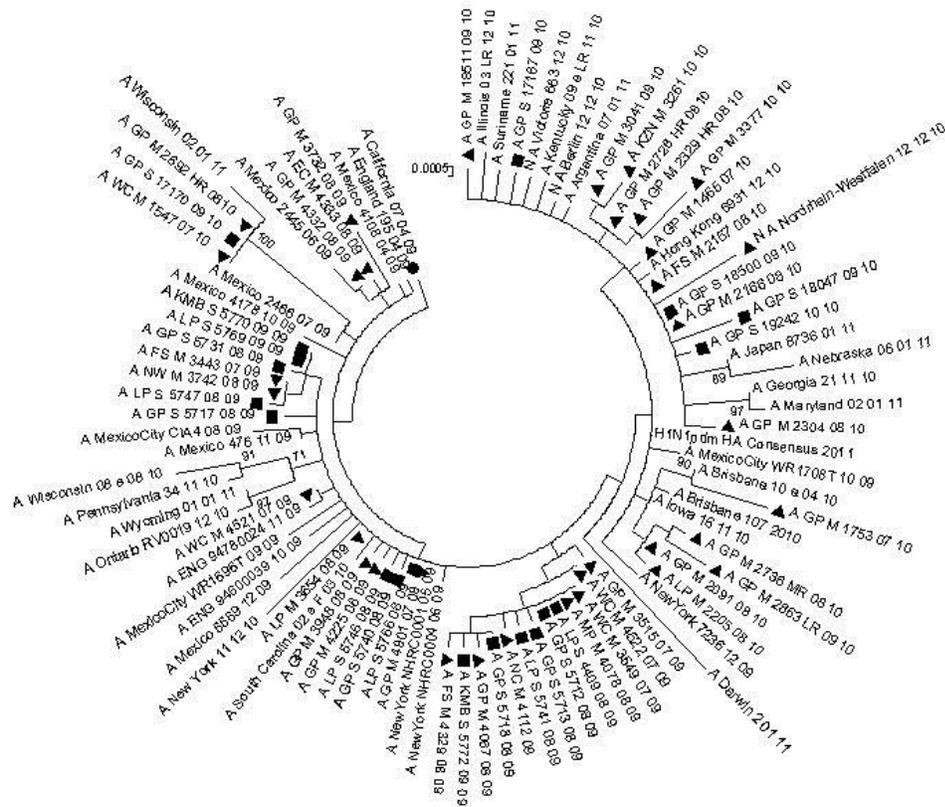
Figure 4

Figure 4: An unrooted maximum likelihood tree showing the evolutionary relationship between South African strains identified in patients with ILI (indicated by triangle) and severe disease (indicated by square) relative to the current vaccine strain, A/California/07/09.

Figure 5: Amino acid changes, from position 8 to 327 of the HA1 region, identified in South African strains from patients with ILI (M) and Severe (S) disease between 2009 and 2010. The D222G mutation identified in Norway is indicated by a grey bar while two clusters of mutations identified in South Africa are indicated in open bars by the arrows in the alignment at S168T and Q294H.