Influenza A(H1N1)pdm09 M and HA segments sequences from Rio Grande do Sul, Brazil

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Key words: Pandemic influenza A (H1N1), Brazil, HA segment, M segment, Rio Grande do Sul

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Abstract

Influenza virus is one of the most critical viruses in epidemiology. The 2009 pandemic was caused by a reassortment of the human-avian-swine virus with 8 RNA segments responsible for all virus proteins. Segment 7 codifies for matrix proteins M1 and M2. These proteins use to show low mutation rate since the matrix is fundamental for virion encapsidation and ion channels formation. On the other hand, HA, segment 4, is one of the most important segments for virulence and for this reason is more studied. Brazil showed many cases just before 2009, from 2011 to 2015, especially in the Rio Grande do Sul State. Two hundred samples obtained during the pandemic were used for amplification and sequencing, reaching 19 M segments amplification and 17 HA amplification that were sequenced. Sequencing of M fragment showed that RS has a different virus origin: Eastern Asia, Western Europe, USA and Central America (Mexico and Nicaragua). All sequences showed amantadine resistance (S31N) and one was out of the phylogenetic tree (Brazil/RS-3335/2009) due to high mutation. RS-3335 is the only sample obtained from a patient that came to die. Many of migration of birds to RS are from Europe, Asia, and USA which could explain this rate of mutations. Insertions and deletions were found in M1 protein of these samples. HA sequences showed worldwide spread and less diversity than M sequences in this work. The most

divergent sample was Brazil/RS-3093/2009 that showed mutations in the sialic acid ligation site.

Introduction

Influenza virus is an enveloped RNA virus, surrounded by a lipid bilayer that belongs to *Orthomyxoviridae* family. This virus is responsible for the most significant pandemics worldwide and is first classified into A, B or C types, the last two less common types. Influenza A is divided according to antibody response for the combination between hemagglutinin (HA) and neuraminidase (NA) proteins, called "H" and "N" for this classification, respectively. There are at least 16 serotypes of H and nine serotypes of N enabling 144 combinations. Segments H1, H2, and H3 combined with N1 and N2 serotypes are more common in humans (Lynch and Walsh 2007). In 2013, a new influenza A (H7N9) was described (WHO 2013). From these serotypes, H1N1 was responsible for 1918 (Spanish flu) (Mills *et al*. 2004) and 2009 (Swine flu) (Schnitzler and Schnitzler 2009) pandemics.

The 2009 pandemic H1N1 (pH1N1) was first described in March, in Mexico; in the cities of Sao Luís do Potosi and Oaxaca. After, on April 17, 2009, it reached the United States of America spreading quickly around the world reaching almost nine thousand deaths in November of that year (Smith *et al*. 2009). In Brazil, the first case was on April 24, in Sao Paulo. Almost at the same time, there was one more case in Sao Paulo, one case in Rio de Janeiro and one case in Minas Gerais, all southeastern states and all patients coming from Mexico (three patients) and USA (one patient). The first incident of death occurred in the Rio Grande do Sul, the southernmost state of the country, on July 26. The patient was a truck driver that was in Argentina for seven days. The Rio Grande do Sul had the highest number of cases and deaths, especially during autumn and winter periods.

H1N1 is a virus consisting of eight RNA segments encoding eleven genes and proteins: RNA polymerase basic proteins (PB1, segment 1; PB1-F2 and PB2, segment 2), RNA polymerase (PA, segment 3), hemagglutinin (HA, segment 4), nucleocapsid protein (NP, segment 5), neuraminidase (NA, segment 6), matrix proteins (M1 and M2, segment 7) and nonstructural proteins (NS1 and NS2, segment 8) (Ghedin 2005). HA and NA are the most studied proteins in influenza resistance and virulence since these proteins are involved in the host immune response and are more likely to mutate. Matrix protein (MP) is divided into two proteins: M1, which is inside the envelope and interacts with genome synthesizing new viral fragments; and M2 that synthesizes ionic channels that provide the interaction between its inner and the environment, mainly when the virus is

producing HA and viral desencapsidation. Unlike HA and NA, MP typically remains conserved, whereas matrix is an essential part of the virus.

One copy of each segment is packaged during viral assembly in one virion particle. PB2, PA, NP and M segments are the most critical segments in packaging (Gao *et al*. 2012). According to Gao *et al.*, (2012), Amorim *et al.* (2011) and Marsh *et al.* (2008) PB1, HA, NA and NS play non-essential roles during genome assembly and may be more permissive toward reassortment then PB2, PA, NP and M. The matrix segment has become more studied since these discoveries. This study aimed to obtain more data about 2009 pandemics in the Rio Grande do Sul State, Brazil, through M and HA sequence analysis and the relationship between symptoms. Brazilian epidemiological data was also used to understand some results.

Materials and Methods

Samples and sequencing

Nasopharyngeal aspirate samples were collected during 2009 outbreak, in the Rio Grande do Sul State, Brazil (Veiga *et al*. 2012) Ethical issues were approved by the Research Ethics Committee of Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). Identification of pH1N1 was conducted at Central Laboratory of the State (LACEN) using Real-Time PCR (Veiga *et al*. 2012). RNA extractions were performed at Universidade Luterana do Brasil (ULBRA) using NewGene Extraction Kit (Simbios Biotecnologia) and handled at Universidade Federal de Ciências da Saúde de Porto Alegre (UFCSPA). The cDNA library was constructed using SuperScript III Reverse Transcriptase according to the manufacturer (Life Technologies). Samples were stored at -80°C.

Many approachs were used to amplify HA and M segments as can disposed in table 1. Conventional PCR was conducted using KAPA 2G Robust HotStart ReadyMix (Biosystems, Boston, USA). Amplicons were treated with Shrimp Alkaline Phosphatase and Exonuclease I, marked with Big Dye Terminator v3.1 kit Cycle Sequencing Kit (Life Technologies) and sequenced at ABI 3130 (Life Technologies). Sequences were constructed and analyzed using Staden 2.0 for Windows using Pregap4 software (Staden 1996). First, all H1N1 sequences were collected from Influenza Research Database (http://www.fludb.org/) and aligned using the online software MAFFT with FF-NS-2 strategy (http://mafft.cbrc.jp/alignment/server/), and a rough tree was constructed. The most similar sequences were selected for a new alignment. After that,

sequences from this work were compared to BLAST (Basic Local Alignment Tool). All matches obtained in this search were used for a new alignment using MEGA 7 software (Tamura et al., 2016) using MUSCLE.

Phylogenetic analysis and map construction

The phylogenetic tree was constructed with MEGA 7 software (Tamura *et al*. 2016), Neighbor-Joining (NJ) (Saitou and Nei 1987) with statistical method of Maximum Likelihood (ML) (Tamura *et al*. 2004). Trees were exported as Newick trees to iTOL online software (Letunic and Bork 2011) to select subtrees to be used for new alignments. Shared phylogenetic trees can be found at iTOL page, login fernandamatias. To construct an interactive map, all sequences were distributed in excel according to its similarity and the clade they belong. This approach would provide a higher view of sequences analyzed in this paper. Information about GenBank accession number, host, strain, influenza type, the location of samples was added to make a more concise and complete map. Subtrees selected created a map of Rio Grande do Sul samples and worldwide more similar samples. The interactive map was constructed using the online platform Google My Maps (https://www.google.com/mymaps/).

Epidemiological Influenza information

During the 2009 influenza pandemic, 218 pandemic influenza samples from Rio Grande do Sul (Veiga *et al*. 2011) were analysed for ARI symptoms (fever, cough, chills, dyspnea, sore throat, arthralgia, myalgia, conjunctivitis, rhinorrhea, diarrhea), and comorbidities (cardiopathy, pneumonia, renal complications, immunodepression, chronic metabolic disease) using Fisher's Exact test to evaluate relationships between them. Only values with $p<0.05$ were considered statistically significant. Gephi 0.9.1 software (Bastian *et al*. 2009) was used to understand the most frequent and most related symptoms. Another approach was to analyze Brazilian online bulletins of influenza provided by Brazilian Health Ministry (Brasil 2012a; Brasil 2012b; Brasil 2013; Brasil 2014; Brasil 2015) to understand the epidemiology of influenza in Brazil from 2009 to 2015. These bulletins were used to distinguish the number of cases and deaths from 2009 to 2015 in different regions of the country (Table 5).

Results

Genetics analysis

Several primers were tested for partial amplification of M and HA segments, as showed in Table 1. First positive results for M segments were obtained using MF1 and MR1 primers having a 700 bp fragment, but from all samples, just three were amplified. The second approach using MF2 and MR2 provided only seven amplifications of 627 bp fragment, which was not a good result yet. Phylogenetic analysis of these amplifications showed a highlighted clade when compared to samples available on genomics databases. It was necessary to obtain a new amplification covering the largest possible area of the segment since that the highlighted clade could be an artifact of the amplified region and small sample size. In this way, it was obtained the third approach trying to achieve complete M segment amplification. From all primer suggested by John Craig Venter Institute (JCVI), ten were synthesized, and combined led to nine pairs that were tested (Table 3). Combinations and number of amplified samples are summarized in Table 3. From all M sequences obtained, only 19 were overlapped creating a contiguous consensus sequence. For HA sequence, the first approach resulted in five positives using HAF1 and HAR1, but second path using HAF2 and HAR2 gave 17 amplifications around 700 bp. Primers from JCVI were used as well but showed no positive amplification in these samples.

M sequences obtained from the Rio Grande do Sul (RS) samples received Genbank accession number KU143688-KU143706 and were compared to all sequences available in genetic databases until October 2014. First, RS sequences were aligned to H1N1 sequences, human or not, open in Influenza Database Research, disregarding the year of Influenza virus sequence. Sequences more like sequences obtained in this work were chosen and retrieved. After that, RS sequences were analyzed one by one in Blast searching for more similar sequences available in this database. With these two approaches, 466 sequences were obtained and compared to RS sequences. All Brazilian sequences were included. The phylogenetic tree showed few branches (red circles) with RS sequences (highlighted in yellow) (Figure 1). Two RS sequences were outgroup in this analysis, Brazil/RS-3335/2009 and Brazil/RS-3504/2009. Seventy-four sequences, including 19 RS sequences, were select for a new alignment and generation of a new phylogenetic tree (Figure 2). The only significant difference in all sequence was obtained in sample Brazil/RS-3335/2009 which patient became to death. From Brazil, there was the only similarity to three another RS sequences and one from Sao Paulo, but only two of them were related to M sequences of these work. This phylogenetic tree was used to generate the interactive map to assess the most similar sequences associated

with the result of this study, (https://www.google.com/maps/d/edit?mid=zQfm5yljIn54.k7PW6v6h1pJI&usp=sharing). This map showed the position of samples obtained in the Rio Grande do Sul State, almost all from Northeast of the State, some near Argentina (Figure 3). From all 60 Brazilian sequences, only two swine sequences (Figure 3, Black Square), Brazil/G2P2/2013 and Brazil/G2P1/2013, both H1N2, from RS were directly related to Brazil/RS-3538/2009 and Brazil/RS-3093/2009 sequences. Another significant data obtained was the global position of most similar sequences linked to sequences of this study that were from USA, Mexico, Western Europe and Eastern Asia (Figure 4). Interactive map shows that from group 14 there is no RS sequence against similar sequences. Most significant changes in amino acids composition were in M1 protein (Table 3). For the construction of M1 table, Brazil/RS-3335/2009 and Brazil/RS-3504/2009 sequences were taken out since they were too much different from California/04/2009, being more like Guangdong/55/2009. This approach was not necessary when comparing to M2 protein. The M1 protein showed insertions, deletions, and substitutions. Brazil/RS-1892/2009 and Brazil/RS-2009/2009 showed many similarities including substitutions in G73A, E197T, Q198T; insertion of R in position 74/75; K in position 197/198, and S in position 200/201. Brazil/RS-2656/2009 showed substitutions in A96T, S196R, Q198T; deletion of E in position 197 and insertion of S in position 200/201. Brazil/RS-2905/2009 showed only one substitution: N91Q. Brazil/RS-3538/2009 showed two substitutions at the beginning of the protein: K47T and P54Y. M2 revealed more similarities than M1. All RS M sequences contain the signature mutation S31N in M2 that might confer amantadine resistance. Sample Brazil/RS-3435/2009 showed the highest number of substitutions: Q77N, D85G, V86C, F91L. Brazil/RS-2014/2009, Brazil/RS-2656/2009, and Brazil/RS-3335/2009 showed one amino acid substitution each: E95K, R54L, I94K, respectively.

Seventeen HA sequences obtained from the Rio Grande do Sul (RS) samples received Genbank accession number MG784976-MG784982 and MG785397-MG785406. These sequences were compared to all sequences available at Influenza Database Research until July 2016, disregarding the year of Influenza virus sequence or the host. The most identical sequences, to those obtained in this work, were chosen and retrieved getting 796 sequences. All similarities obtained were for 2009, 2010 and 2011 sequences, and only three swine-like sequences. Three phylogenetic trees were constructed until

obtaining the most similar sequences, getting a total of 258 sequences. The third phylogenetic tree showed only four groups (Figure 5) and was used to construct the interactive map (https://www.google.com/maps/d/u/0/edit?hl=pt-BR&mid=1zn3GhGcRZHYGyct__jIsERPA0ZDQ5Nj4&ll=5.411776974245612%2C0&z=2). The map obtained (Figure 6) showed the worldwide spread (Figure 6A) of HA segment compared to HA sequences of this work and high similarity to another Brazilian (Figure 6B) HA. Distribution of samples were in the majority from the middle to the east of RS State (Figure 6C). There are only amino acids substitutions in these sequences when compared to California/04/2009; Brazil/RS-3900/2009 Q32E, Brazil/RS-3466/2009 A38E, Brazil/RS-5377/2009 D65E, Brazil/RS-2529/2009 G105E, Brazil/RS-5081/2009 T113K, Brazil/RS-2014/2009, Brazil/RS-2584/2009, Brazil/RS-3869/2009 Q136H. Brazil/RS-3093/2009 was the most different sequence showing N137D, I138M, H139Q, I141T substitutions. Almost all sequences showed substitution I164V, except for Brazil/RS-5377 that showed I167V. The only patient death occurred in Brazil/RS-3222/2009 that showed only one substitution (I164V).

Epidemiology analysis

Although pH1N1 presents no danger in the world, in Brazil this virus is still monitored since it stayed between circulating viruses and showed higher mortality over the years. In 2014, there was an increase in cases of H3N2, overcoming cases of pH1N1 in all regions of Brazil. However, in percentage, the number of deaths was lower than pH1N1. The virus Influenza A H3N2 presented a proportion of deaths in 2013 and 2014 around 9% while the Influenza A pH1N1 virus showed mortality of 20.6% in 2013 and 35% in 2014 (Table 6). The number of cases in Southeast and South of Brazil, highlighting states of South and São Paulo, from 2009 to 2015 is showed in Table 6. It is observed that Paraná has the highest number of cases in the south, which remained in 2010. In 2010, Rio Grande did Sul (RS) has reported no evidence of pH1N1. In 2011, Rio Grande do Sul shows cases of this virus again, becoming the state with the highest number of cases, unlike other states where the numbers were declining from 2009. In 2012, Santa Catarina, the state just above the Rio Grande do Sul, stands out as the state with the most substantial number of cases, surpassed in 2013 by Sao Paulo, with a decrease of cases in the three states of the South.

According to symptoms observed in RS A(H1N1)pdm09 epidemiological data (Figure 7) chill was the most frequent and more related symptom. Chill showed a direct

relationship between fever, the group of symptoms related to each other arthralgia-myalgia; throat-arthralgia, conjunctivitis, and rhinorrhea. The second frequent symptom was rhinorrhea pertaining to chill, conjunctivitis, and cough. There was no significant relationship between metabolic disease, immunosuppression, heart disease and lung disease to other symptoms.

Discussion

Pandemic H1N1 (2009) is a reassortment between North American triple-reassortant classical swine virus and Eurasian avian-like swine influenza virus (Morens *et al*. 2009, Zimmer *et al*. 2009) and in this analysis, it is crucial to consider avian and swine influenza by themselves. Strain California/04/2009 was chosen for comparison because it is a model of transmission (Munster *et al*. 2009) and was used for annotation as well.

The small number of amplified samples and an even lower number of overlapping sequences can be explained by the problem of maintenance of samples as discussed by Veiga *et al.* (2012). During the crisis of 2009, the number of patients throughout the state of Rio Grande do Sul was considerable, and there was no proper way of collecting and storage samples (Veiga *et al*. 2012).

Samples Brazil/3335/2009 and Brazil/3504/2009 showed the highest similarity to Guangdong/55/2009 (HQ011424). When this sequence was analyzed in IRD, it was found another 12 similar sequences of segment M. One of these sequences was KC876544, H3N2, from Argentina. Since Rio Grande do Sul borders Argentina, many of the cases in the State were from this country that could explain the diversification of this segment in RS. All RS sequences, just like HQ011424 and KC876544, showed S31N substitution related to decreased resistance to amantadine (Lan *et al*. 2010). Although NA segment is important for influenza virus virulence, it is not crucial for genome virus assembly (Gao *et al*. 2008). According to Gao and colleagues, (2012), virus lacking segments PB2, M or NP have low growth and are easily lost during passages in eggs, since these segments seem to recruit other segments during genome packaging. Silent mutations in M segment can result in packaging defect in all other segments (Hutchinson *et al*. 2008). M1 is an import protein in virus assembly. This protein is responsible for association with influenza virus RNA and ribonucleoprotein (RNP) controlling the nuclear export and import of RNP and some functions in transcription inhibition (Baudin *et al*. 1994, 2001; Bui *et al*. 1996, Huang *et al*. 2001; Whittaker *et al*. 1995). Besides, that M1/M2 can change the balance of HA and NA

affecting one or both glycoproteins (Campbell *et al*. 2014). Moreover, M from pH1N1 is essential for virion morphology contributing to HA recognition, and NA increased activity improving virus transmission (Campbell *et al*. 2014). Experiment using guinea pigs demonstrated that strains without pH1N1 M could not transmit the virus while those containing NL + M + NA + HA had great transmission even with a 100 PFU virus dose (Campbell *et al*. 2014). According to van Wielink and colleagues (2012), mutations in M and NP indicated an average decrease in virus titers when combined while mutations in M itself increased virus titer indicating that M alone determined enhanced replication. NS1 is expressed at high levels after infection and facilitates virus replication (Hale *et al*. 2008). One of the goals of NS1 is mRNA splicing of M segment (Robb and Fodor 2012). The increase titers of delNS1 were due to M1 amino acid substitution M86V, a region near to helix six domain, a positively charged surface region (van Wielink *et al*. 2012, Sha and Luo 1997). Adaptation of M-segment mutations could compensate the absence of NS1 protein (delNS1), and these modifications can increase expression of M1/M2 ratio even if M2 has silent mutations and M1 with silent or nonsilent mutations affecting M1 mRNA splicing efficiency (van Wielink *et al*. 2012). M1 and M2 proteins can alter NA Vmax through alterations in NA incorporation, distribution or presentation at the virion surface. It happens since NA cytoplasmic tail interacts in transmembrane regions with M1 protein (Campbell *et al.* 2014, Ali *et al*. 2000, Barman *et al*. 2001, Mitnaul *et al*. 1996).

In matrix segment, it was found that substitutions in M1 and M2 were significant in virulence. M2 has demonstrated resistance to amantadine and rimantadine if there is S31N substitution (Ilyushina *et al*. 2005), all RS samples showed this signature. This substitution is present in Guangdong/55/2009 and swine/Jangsu/48/2010 both related to samples analyzed in this work. The swine/Jangsu/48/2010 was related to Brazil/3012/2009, and Guangdong/55/2009 was near to the related Brazil/RS-3335/2009 and Brazil/RS-3504/2009 in the first tree construction with all 466 sequences using Neighbor-Joining phylogenetic test and directly related to Brazil/RS-3504/2009 in the second phylogenetic tree. The patient who provided the sample Brazil/RS-3335/2009 was the only one that came to death, and showed the highest divergence to all sequences, just like samples one and two from Beijing. Substitution in 16 and 55 positions of M2 showed enhanced transmission in humans. Sample Brazil/RS-2656/2009 showed substitution near 55 positions, in R54L position, which could be involved in increased transmission as well. Fan and colleagues (2009) found that M

gene from H5N1 avian influenza viruses contributed to virulence difference in mice while other genes could attenuate pathogenicity. It is important to consider due to the geographical position of RS. There is a presence of influenza A H3N2 M segment related to pH1N1 M segment from the Rio Grande do Sul due to the representativeness of this virus in reassortment of 2009 pandemic, just like swine/texas4199-2/1998 (H3N2) (Chou *et al*. 2011; Webby *et al*. 2000). The most divergent sequences were obtained in M1 Brazil/RS-3335/2009 and Brazil/RS-3504/2009. Many differences in sequences were obtained in M1 Brazil/RS-1892/2009, Brazil/RS-2009/2009, Brazil/RS-2656/2009, and M2 Brazil/RS-3435/2009. Sequences Brazil/RS-2905 and Brazil/RS-3538/2009 showed one and two substitutions in M1, respectively, and Brazil/RS-2014/2009, Brazil/RS-2656/2009, Brazil/RS-3335/2009 showed one substitution in M2 each. M1 and M2 could have different morphology, since some of the samples showed signatures near those related to virion morphology, especially in M1 sequence. According to Elleman and Barclay (2004), some of this amino acids morphology determinants in M1 were 41, 95, 218, while Bourmakina and Garcia-Sastre (2003) found M1 95 and 204 as determinants. Roberts *et al*. (1998) found that M1 and M2 were responsible for virion morphology, including 41 amino acids from M1. Campbell *et al*. (2014) found 13 amino acid difference including 41, 207, 209, 214 in M1, a similar model of Elleman and Barclay, but included the difference of 14 amino acid in M2.

The most similarity of HA segment was obtained with Brazil/RS-2529/2009 to CY075275 from Chile, Brazil/RS-5081/2009 to JN171873 from Quebec, and Brazil/RS-5377/2009 to CY070245 from England. CY075275 and JN171873 are similar to GQ166223 from China, sample that showed a high level of replication and mutation of inosine 32 positions (Xu *et al*. 2011). The only sample with a mutation in this site was Brazil/RS-3900/2009 but for glutamic acid. Strains Brazil/RS-2014/2009, Brazil/RS-2584/2009, Brazil/RS-3093/2009 (sequence MG784980) and Brazil/RS-3869/2009 showed substitutions in the sialic acid ligation site (Al-Maihdi 2007). The most divergent sequence was MG784980 with four mutations in the sialic acid ligation region.

Classical swine influenza virus (SIV) is like 1918 pandemic virus and remained antigenic and genetically conserved in the USA until the introduction of H3N2 in 1998 (Vicent *et al*. 2008). In Europe, an avian-like virus H1N1 was predominant in swine until the introduction of H3N2 reassortment in the 1980s (VanReeth 2007). Pigs have a

receptor for both human and avian influenza virus becoming an essential player for interspecies transmission. In Brazil, pH1N1 is established in swine populations and may become endemic in the country (Rajão *et al*. 2013). In an experiment using California/04/2009 (H1N1) and Swine/Texas/4199-2/1998 (H3N2) showed that only viruses harboring M segment from California/04/2009 exhibited high transmissibility through the air in guinea pigs (Chou *et al*. 2011). California/04/2009 is highly transmitted through aerosol route while Swine/Texas/4199-2/1998 did not show this capability. These findings could explain the late substitution of pandemic H1N1 to seasonal H3N2 in RS. Epidemiological data (Brasil 2012a; Brasil 2012b; Brasil 2013; Brasil 2014; Brasil 2015) showed a decrease in cases of pH1N1 probably by competing with H3N2. Apparently, the H3N2 virus is more infectious than pH1N1, since it has affected more patients that the pandemic virus. This competition could lead to the extinction of the pandemic virus in Brazil. The most common symptoms were chill and rhinorrhea, but not the fever that is usual in virus infection. Fever is almost always associated with an immunological response to infection, and this result could indicate why H1N1pdm09 spread worldwide so quickly.

Brazilian SIV HA and NA network suggested a common origin for all viruses isolates, irrespective of the region or host (Rajão *et al*. 2013) which is not the same for H1N1pdm2009. In a previous study, Sant'Anna *et al.* (2013) found that most of the Brazilian influenza A pH1N1 sublineages from the Rio Grande do Sul belong to clade 7 (Nelson 2009) assuming that multiple sublineages were introduced in Brazil. M sequences of this work showed not only different origins but also a new clade origin which happened to HA as well with Brazil/RS-3093/2009. As M origin is birds, the high divergence in sequences could be due to migratory birds that allow new recombination of the virus in bird and transmission to humans. The Rio Grande do Sul is also an import place for bird birth and migration during summer and winter. According to Morrison and Ross (1989), there are representatives of *Calidris sp*., *Pluvialis sp*., *Tryngites sp*., *Sterna sp*., *Cygnus sp*., *Dendrocygna sp*., *Gallinula sp*., *Plegladis sp*., *Netta sp*., *Coscoroba sp*. among others. It is possible that M segment came from Eurasian avian-like swine and had the natural selection or even, as discussed by Sant'Anna *et al.* (2013), founder effect. Thinking of that, as proposed by Ozaki *et al.* 2014, specific host cell phenotypes may differentially influence virus replication since reassortments between influenza A H6N1 and influenza A H6N2 PB2 and M repressed replication in the chicken trachea. In another study, avian mutations in M segment

increased virulence in mice since M1 protein contributes to the virulence of H5N1 avian influenza viruses (Fan et al. 2009).

HA sequences (classical swine segment) showed less divergent sequences then M sequences (avian-like segment). It is important to say that Influenza Database Resource had until January 16, 2018; 19,018 M and 34,565 HA sequences in its system. According to this data, HA has almost the double of M sequences in the genomic database and this difference could provide the few distance and similarities of HA sequences, which didn't happen to M sequences.

Epidemiological data along with the molecular analysis of samples obtained in 2009 may explain the increased number of cases in the Rio Grande do Sul from 2011 to 2012 and consequently the increase in cases in 2012 in the states of Santa Catarina and Paraná, culminating in the highest number of cases in Sao Paulo in 2013. São Paulo is the most populous state in the country and shows some winter characteristics of the South, such as the change in temperature, that together to air pollution could explain the high number of cases in the State. It is possible that differentiated viruses, and grouped in a clade apart from the phylogenetic tree have remained somehow in the state and turned more virulent form from 2011. Like Rajão *et al*. (2017) showed, there are many reassortments between H1N1pdm09 and H3N2 in pigs. These reassortments include at least one segment of H1N1pdm09 in H3N2, especially M segment, and HA was stable, as we show in this work for humans. As discussed above, Rio Grande do Sul, just like Santa Catarina, is a bird migration area, which may have influenced the maintenance of this virus in the country, as well as new arrangements of M segment since M segment comes from birds or pigs. Santa Catarina has most of the pig breeding sites of the country and could make reassortments between birds and pigs.

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Conflict of interest

No conflict of interest declared.

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Table 1: Primers used in this work.

Primer Name	Primer Sequence	Segment	Approach	Reference
MF1	CGAGGTCGAAACGTACGTTC	M	1	This work
MR1	CAGACCAGCACTGGAGCTAG			
MF2	AGATCGCGCAGAGACTGGAAAGT		2	This work
MR2	TCTTTCAGACCAGCACTGGAGCTA			
MP1F	TGTAAAACGACGGCCAGTAGCRAAAGCAGG		3	JCVI
MP5F	TGTAAAACGACGGCCAGTAAAGCAGGTAGATRTT			
MP289F	TGTAAAACGACGGCCAGTGAYCCRAAYAACATGG			
MP388F	TGTAAAACGACGGCCAGTGGTGCACTTGCCWG			
MP622F	TGTAAAACGACGGCCAGTGCNGAGGCYATGGA			
MP407R	CAGGAAACAGCTATGACCTRCARCWGGCAAGTGCACC			
MP502R	CAGGAAACAGCTATGACCTGCTGKGARTCAGCAATYTG			
MP740R	CAGGAAACAGCTATGACCCCTGYAAATTTTCAAGAAGATC			
MP1010R	CAGGAAACAGCTATGACCTTTTTACTCYAGCDCTATG			
MP1027R	CAGGAAACAGCTATGACCAGTAGAAACAAGGTAGTTTT			
HAF1	CATGTCCTCATGCTGGAGC	HA	1	This work
HAR1	CTCGTCAATGGCATTCTGTG			
HAF2	ACCCAAAGCTCAGCAAATCCTACA		2	This work
HAR2	TCTTCAGGTCGGCTGCATATCCT			

Table 2: Strategy of primers used in this work.

Primers	Amplicon size	Strategy	Result
HAF1 + HAR1	~700 bp	1	Few positives
MF1 + MR1	~700 bp		
HAF2 + HAR2	627 bp	2	Few positives, more than strategy 1
MF2 + MR2	625 bp		
MP1F + MP407R	406 bp	3	Negative
MP1F + MP740R	739 bp		Negative
MP5F + MP502R	497 bp		Negative
MP289F + MP740R	451 bp		Positive
MP388F + MP1010R	633 bp		Positive
MP388F + MP1027R	639 bp		40 positives
MP622F + MP1027R	405 bp		Positive
MF1 + MP740R	739 bp		30 positives

Shadow: primers used to obtain partial M and HA segment.

Table 3: Amino acids substitutions, insertions and deletions in M1 and M2 proteins of RS influenza virus pH1N1.

Strains	M1											M2						
	Substitutions								Insertions			Substitutions						
	47	54	73	91	96	196	197	198	74/75	197/198	200/201	54	77	85	86	91	94	95
FJ969513 A/California/04/2009	K	P	G	N	A	S	E	Q	0	0	0	R	Q	D	V	F	I	E
KU143699 A/Brazil/RS-1805/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143701 A/Brazil/RS-1892/2009	*	*	A	*	*	*	T	T	R	K	S	*	*	*	*	*	*	*
KU143695 A/Brazil/RS-2009/2009	*	*	A	*	*	*	T	T	R	K	S	*	*	*	*	*	*	*
KU143697 A/Brazil/RS-2014/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	K
KU143694 A/Brazil/RS-2543/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143691 A/Brazil/RS-2656/2009	*	*	*	*	T	R	deletion	T	0	0	S	L	*	*	*	*	*	*
KU143689 A/Brazil/RS-2763/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143703 A/Brazil/RS-2905/2009	*	*	*	Q	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143706 A/Brazil/RS-3012/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143696 A/Brazil/RS-3082/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143700 A/Brazil/RS-3093/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143688 A/Brazil/RS-3435/2009	*	*	*	*	*	*	*	*	0	0	0	*	N	G	C	L	*	*
KU143702 A/Brazil/RS-3466/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143698 A/Brazil/RS-3538/2009	T	Y	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143690 A/Brazil/RS-3908/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143705 A/Brazil/RS-5081/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143692 A/Brazil/RS-5377/2009	*	*	*	*	*	*	*	*	0	0	0	*	*	*	*	*	*	*
KU143693 A/Brazil/RS-3335/2009	[redacted]											*	*	*	*	*	K	*
KU143704 A/Brazil/RS-3504/2009	[redacted]											*	*	*	*	*	*	*

* No amino acid change.

Table 4: Amino acids substitutions in HA protein of RS influenza virus pH1N1.

Strains	Substitutions											
	32	38	65	105	113	136	137	138	139	141	164	167
FJ966082 A/California/04/2009	Q	A	D	G	T	Q	N	I	H	I	I	I
MG785398 A/Brazil/RS-1865/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG784979 A/Brazil/RS-2014/2009	*	*	*	*	*	H	*	*	*	*	V	*
MG785405 A/Brazil/RS-2529/2009	*	*	*	E	*	*	*	*	*	*	V	*
MG784978 A/Brazil/RS-2543/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG785399 A/Brazil/RS-2584/2009	*	*	*	*	*	H	*	*	*	*	V	*
MG784976 A/Brazil/RS-2656/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG784980 A/Brazil/RS-3093/2009	*	*	*	*	*	*	D	M	Q	T	-	-
MG785403 A/Brazil/RS-3222/2009	-	*	*	*	*	*	*	*	*	*	V	*
MG784981 A/Brazil/RS-3466/2009	*	E	*	*	*	*	*	*	*	*	V	*
MG785400 A/Brazil/RS-3542/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG785402 A/Brazil/RS-3869/2009	*	*	*	*	*	H	*	*	*	*	V	*
MG785397 A/Brazil/RS-3900/2009	E	*	*	*	*	*	*	*	*	*	V	*
MG785406 A/Brazil/RS-4028/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG785401 A/Brazil/RS-4252/2009	*	*	*	*	*	*	*	*	*	*	V	*
MG784982 A/Brazil/RS-5081/2009	*	*	*	*	K	*	*	*	*	*	V	*
MG784977 A/Brazil/RS-5377/2009	*	*	E	*	*	*	*	*	*	*	*	V
MG785404 A/Brazil/RS-5446/2009	*	*	*	*	*	*	*	*	*	*	V	*

* No amino acid change

- No data available

Table 5: Number of cases and death caused by influenza A pH1N1 in Brazil from 2009 to 2015.

	Year	2009		2010		2011		2012		2013*				2014				2015**			
	Influenza A	pH1N1		pH1N1		pH1N1		pH1N1		pH1N1		H3N2		pH1N1		H3N2		pH1N1		H3N2	
		Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death	Cases	Death
Brazilian region	North	868	50	319	48	2	0	20	7	114	27	6	2	14	7	18	2	2	0	12	3
	Northeast	846	62	152	23	1	0	76	12	116	29	32	2	33	7	26	3	2	0	37	1
	Southeast	12,104	992	120	17	60	6	508	118	2,466	537	203	28	171	66	486	60	19	6	230	35
	South	35,397	789	364	21	112	14	1,884	189	911	139	405	24	96	25	438	30	81	17	208	15
	Center West	1,267	167	18	4	6	1	123	25	121	32	22	5	150	58	74	10	5	3	53	13
	Total Brazil	50,482	2,060 (4)	973	113(11)	181	21(11)	2611	351(13)	3733	768(20.6)	668	61(9.1)	464	163 (35.1)	1,042	105(10)	109	26(23.8)	540	67(12.4)

* Starts co-circulation of H3N2 cases in 2013: 668 cases with 61 deaths; 2014: 887 cases, 82 deaths.

** Data until October 2015.

Table 6: Cases of influenza A pH1N1 from 2009 to 2014 in Southeast and South of Brazil.

Brazilian region	State	Year						
		2009	2010	2011	2012	2013*	2014	2015
Southeast	MG	1810	8	26	134	425	31	5
	ES	110	1	1	0	17	1	0
	RJ	2777	3	5	4	52	22	0
	SP	**7407**	**108**	**28**	**370**	**1972**	**117**	**14**
South	**PR**	**30650**	**344**	**2**	**621**	**353**	**46**	**31**
	SC	**2155**	**20**	**7**	**743**	**225**	**21**	**50**
	RS	**2592**	**0**	**113**	**520**	**333**	**29**	**0**

Brazilian States: MG - Minas Gerais, ES - Espírito Santo, RJ - Rio de Janeiro, SP - São Paulo, PR - Paraná, SC - Santa Catarina, RS - Rio Grande do Sul.

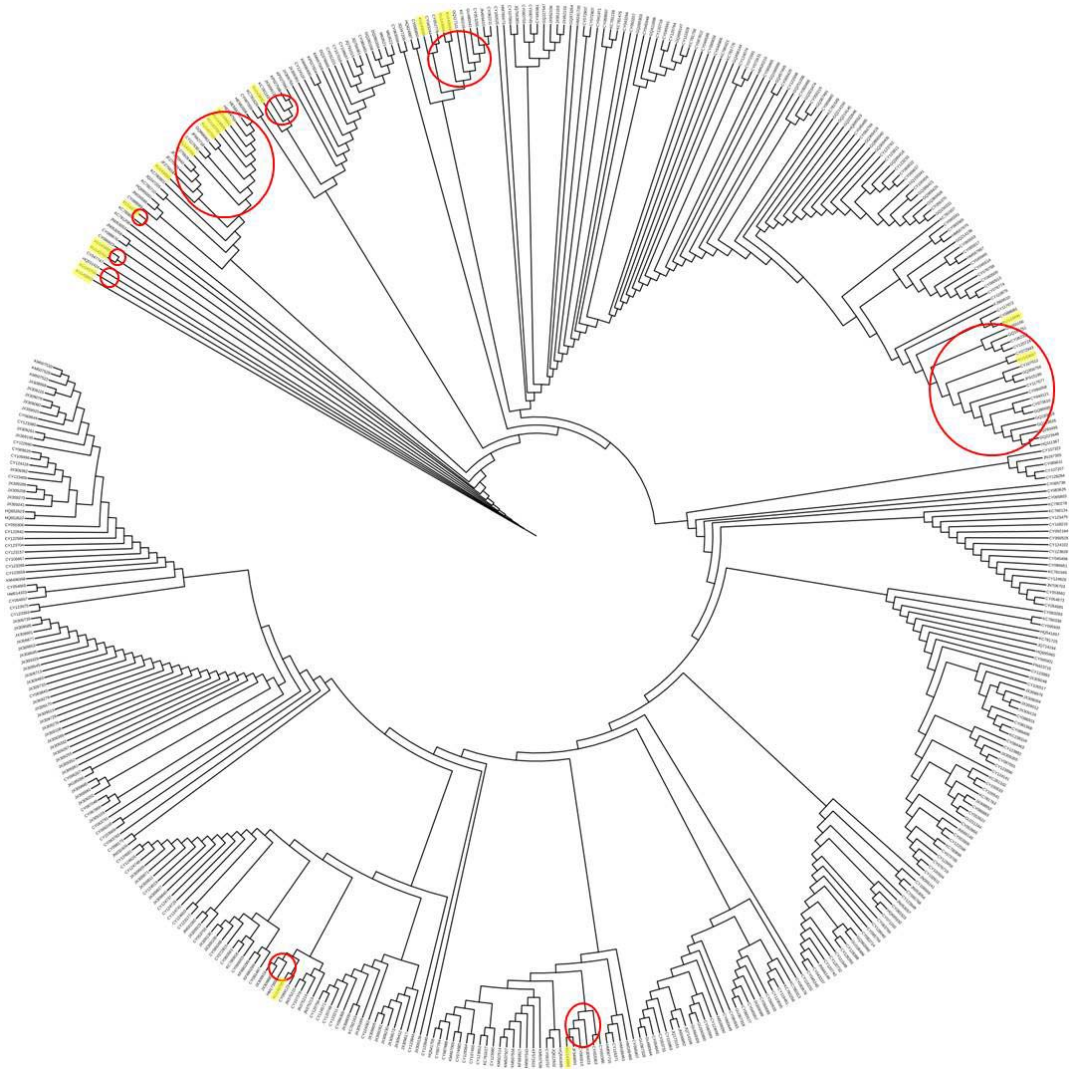

Figure 1: second selection with 466 sequences all over the world. All sequences of the branch with some of the RS sequences (yellow squares) were selected (red circles) to the new alignment.

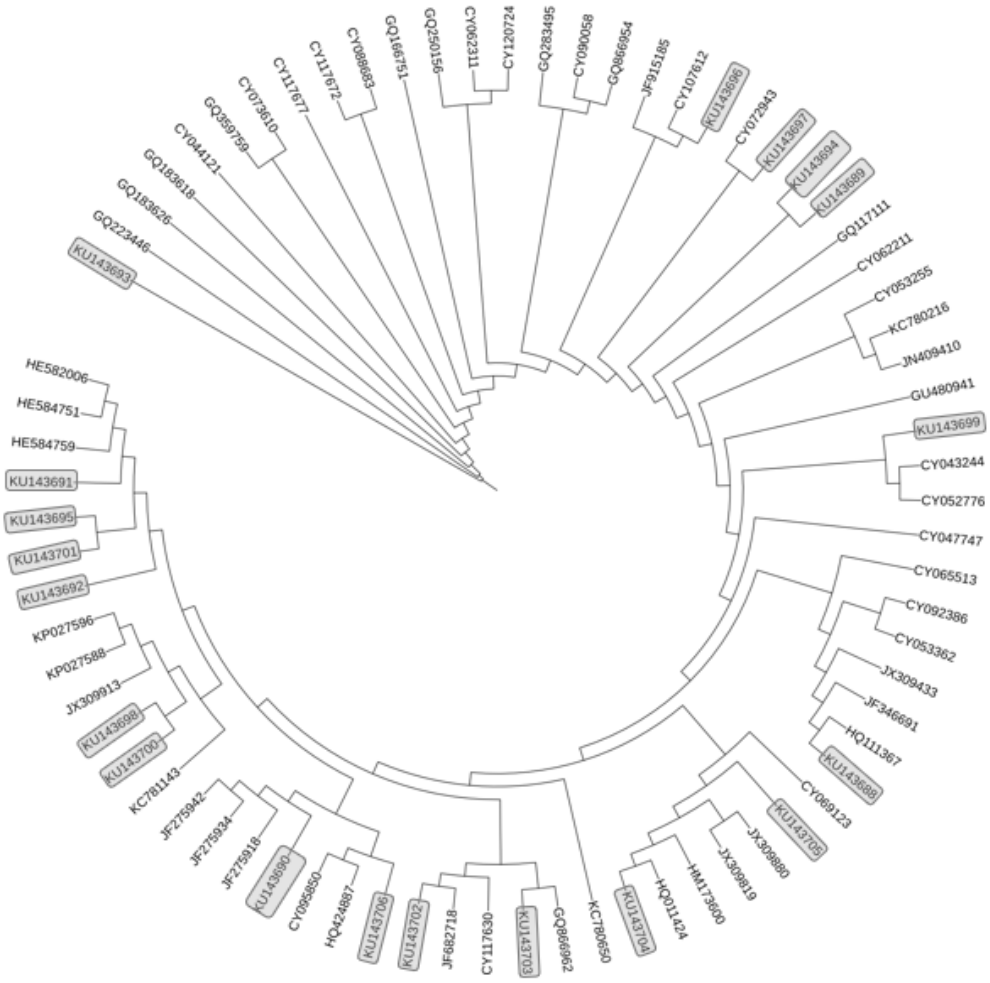

Figure 2: Final phylogenetic tree with 55 of the most similar sequences related to 19 RS sequences (gray squares). The most divergent sequence is KU143693 (Brazil/RS-3335/2009) nearby sequences from Beijing.

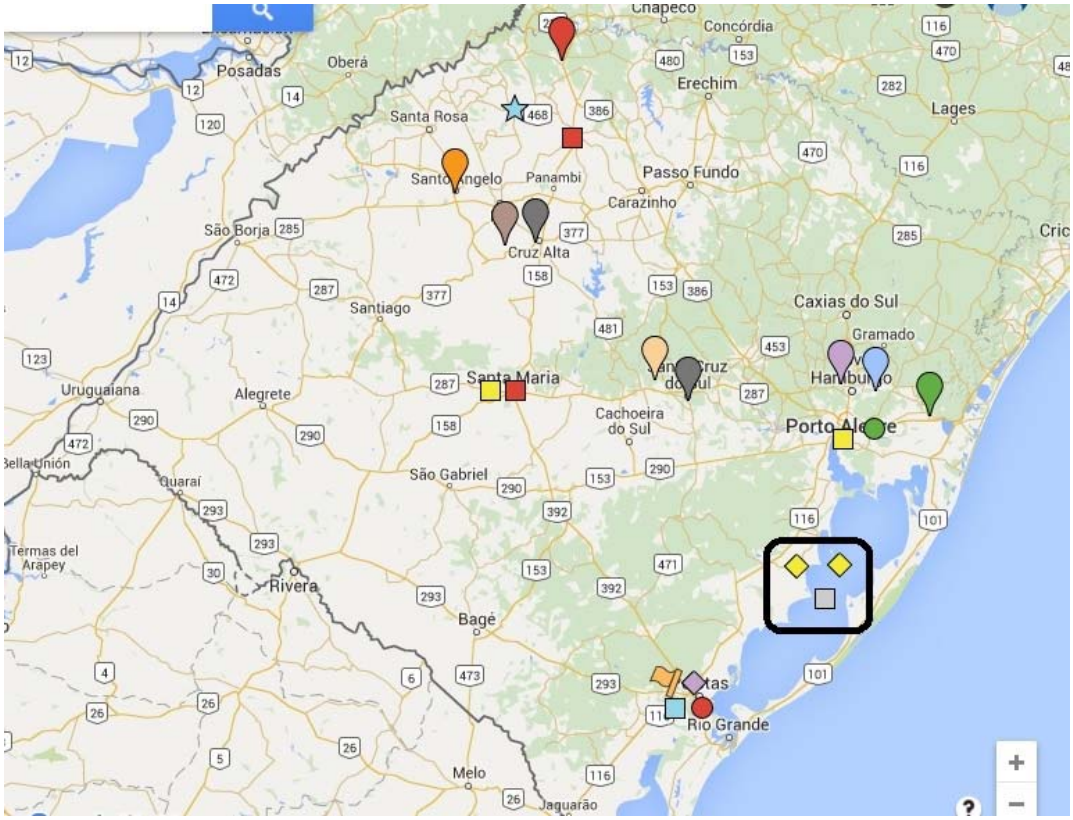

Figure 3: Rio Grande do Sul Map. It is interactive, click the link below and open image. Clicking icons, it is possible to see all information about data. The same color is the same branch. Drops are the first level, squares are the second level, circles are the third level, a rhombus is the fourth level, stars are the fifth level, and flags are the sixth level of the same branch of the tree. It is possible to see more similarity between samples analyzed in this study than from others. There are only three from RS sequences similar to those from theses work (Black Square), and the most similar are from swine and not human as yellow color shows. (https://www.google.com/maps/d/edit?mid=zQfm5yljIn54.k7PW6v6h1pJI&usp=sharing)

Figure 4: World Map constructed using phylogeny tree. It is interactive, click the link below and open image. Clicking on the icons, it is possible to see all information about data. The same color is the same branch. Drops are the first level, squares are the second level, circles are the third level, a rhombus is the fourth level, stars are the fifth level, and flags are the sixth level of the same branch of the tree. It is possible to see that most similarities were obtained to sequences from USA, Mexico, Western Europe, and Southeast Asia.

(https://www.google.com/maps/d/edit?mid=zQfm5yljIn54.k7PW6v6h1pJI&usp=sharing)

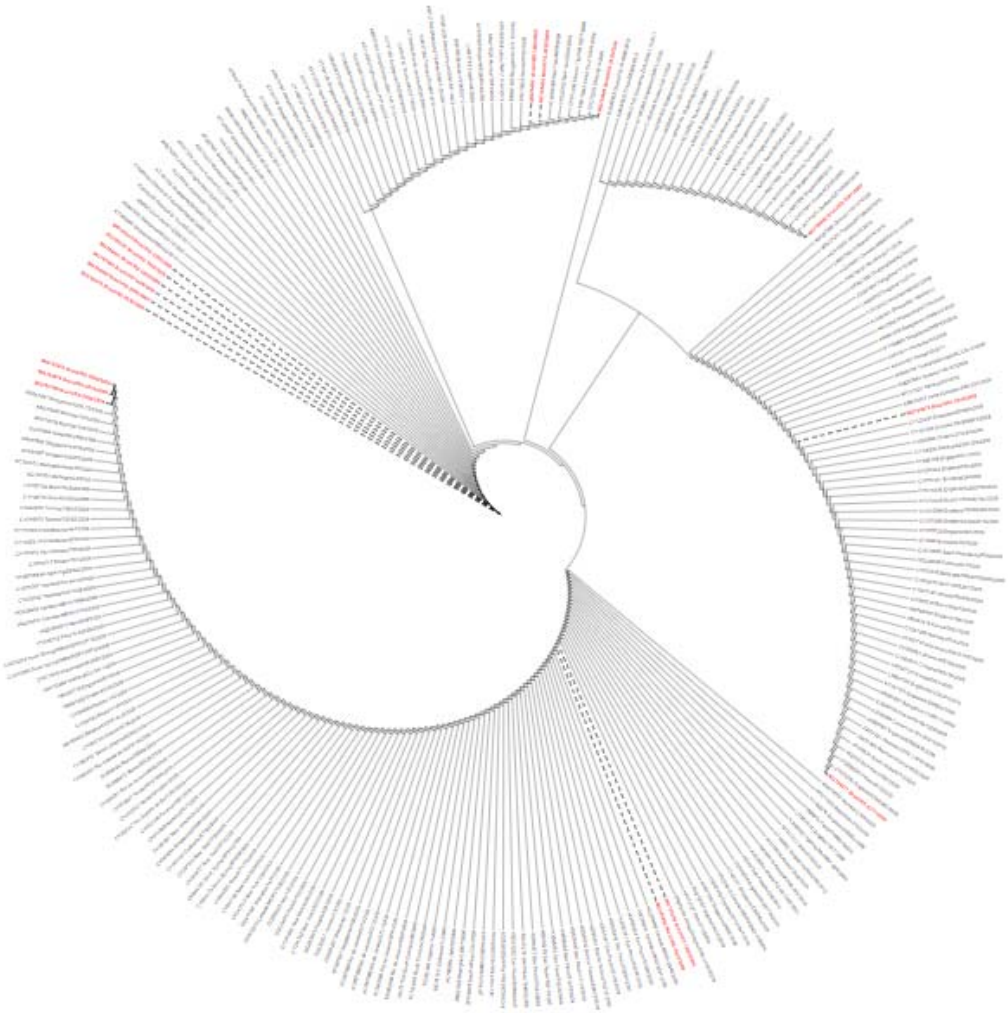

Figure 5: Final phylogenetic tree with 258 of the most similar sequences related to 17 HA RS sequences (red writing). The most divergent sequence is MG784980 showing four mutations which led to the formation of a new clade.

Groenlândia
Islândia
Finlândia
Suécia
HQ228048
CY075480
HM567776
HM189402
Rússia
KM277596
JF789636
Mongólia
China
Japão
GQ200287
HQ239435
CY060750
KC781551
CY071978
CY060694
México
CY071698
Oceano Atlântico Norte
Espanha
GU585420
Argélia
Líbia
KJ133554
Iraque
Arábia Saudita
Afegani
KP317226
KU310626
CY087288
CY075767
KR861651
Níger
Chade
Sudão
KJ026436
KX431201
Tanzânia
KF667892
Indonésia
Papua-Nova Guiné
Venezuela
Colômbia
CY045112
CY120723
MG785399
Chile
Argentina
CY075275
Oceano Atlântico Sul
Angola
Namíbia
JF745829
Madagá
JQ431203
Oceano Índico
Austrália
Nova Zelândia
Google My Maps
A

Caracas
Venezuela
Bogotá
Colômbia
Guiana
Guiana Francesa
Suriname
RORAIMA
AMAPÁ
Quito
Equador
AMAZONAS
PARÁ
MARANHÃO
Fortaleza
CEARÁ
PIAUÍ
CY103925
ALAGOAS
SERGIPE
Salvador
CY045112
Peru
Lima
Brasil
RONDÔNIA
TOCANTINS
MATO GROSSO
CY120723
HQ595252
GOIÁS
Brasília
BAHIA
MINAS GERAIS
ESPÍRITO SANTO
Bolívia
KF612092
MATO GROSSO DO SUL
SÃO PAULO
CY054281
Paraguai
Assunção
CY060452
Chile
MG785399
MG784982
Córdova
Uruguai
Santiago
Buenos Aires
Argentina
CY075275
Montevidéu
B

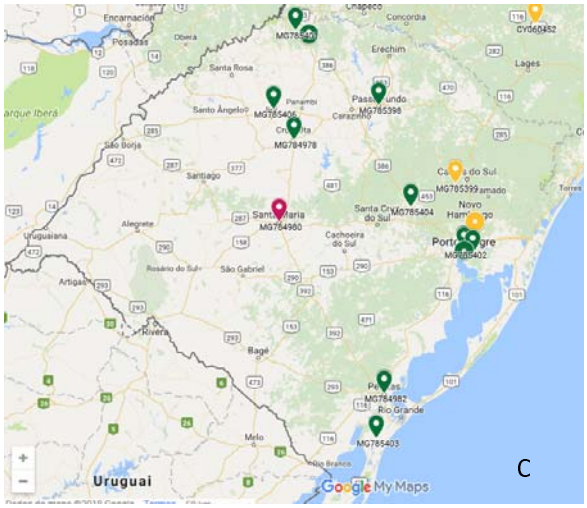

Figure 6: Map of HA most similar sequences obtained from Influenza Research Database to Rio Grande do Sul State (RS). Distribution of HA most similar sequences around the world (A), most similar sequences of Brazil (B) and distribution of samples in RS (C).

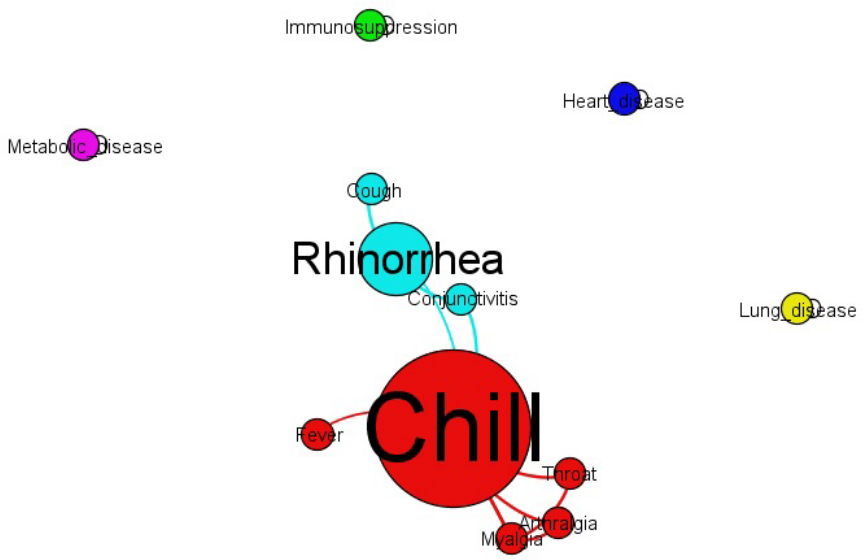

Figure 7: Relationship between symptoms showing chill (biggest red circle) as the most frequent and rhinorrhea (biggest blue circle) as second most frequent and related symptom. Metabolic disease, immunosuppression, heart disease and lung disease didn't show any relation during A(H1N1)pdm09 in Rio Grande do Sul.