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High conservation level of CD8+ T cell immunogenic regions within an unusual H1N2 human influenza variant.

Abstract

Current seasonal influenza vaccines require regular updates due to antigenic drift causing loss of effectiveness and therefore providing little or no protection against novel influenza A subtypes. Next generation vaccines capable of eliciting CD8+ T cell (CTL) mediated cross-protective immunity may offer a long-term alternative strategy. However, measuring pre- and existing levels of CTL cross-protection in humans is confounded by differences in infection histories across individuals. During 2000-2003, H1N2 viruses circulated persistently in the human population for the first time and we hypothesised that the viral nucleoprotein (NP) contained novel CTL epitopes that may have contributed to the survival of the viruses. This study describes the immunogenic NP peptides of H1N1, H2N2 and H3N2 influenza viruses isolated from humans over the past century, 1918-2003, by comparing this historical dataset to reference NP peptides from H1N2 that circulated in humans during 2000-2003. Observed peptides sequences ranged from highly conserved (15%) to highly variable (12%), with variation unrelated to reported immunodominance. No unique NP peptides which were exclusive to the H1N2 viruses were noted. However, the virus had inherited the NP from a recently emerged H3N2 variant containing novel peptides, which may have assisted its persistence. Any advantage due to this novelty was subsequently lost with emergence of a newer H3N2 variant in 2003. Our approach has potential to provide insight into the population context in which influenza viruses emerge, and may help to inform immunogenic peptide selection for CTL-inducing influenza vaccines.

Word count: 240

Keywords: influenza, H1N2, immunogenic, peptides, cross-protection.

Introduction

Strain specific vaccines incorporating the hemagglutinin (HA) and neuraminidase (NA) antigens of influenza viruses are the currently recommended public health measure for influenza prevention [Schultz-Cherry and Jones]. However these neutralising antibody-based vaccines lose their efficacy over time because of antigenic shift or drift in the circulating virus population associated with the accumulation of mutations, or intra-subtype reassortment [Hirst, 1943; Holmes et al., 2005; Lindstrom et al., 1998; Sigel et al., 1947]. Antigenic drift necessitates frequent vaccine updates to ensure sufficient

antigenic match between the HA variants included in the vaccine and those predicted to predominate in the next influenza season. Importantly, seasonal influenza vaccines are not anticipated to protect against major genetic shifts in the virus, or the introduction of entirely novel influenza subtypes as occurred in 2009 [Kelso, 2012]. To overcome these limitations, there is a strong interest in developing next generation influenza vaccines or vaccine-components capable of eliciting broadly cross-protective $CD8^+$ T cell responses. As cytotoxic T lymphocytes (CTLs) primarily target well-conserved internal viral proteins harnessing CTL immunity can potentially afford protection against both known and novel emerging influenza viruses [Hayward et al., 2015; Sridhar et al., 2013; Wang et al., 2015].

This alternative vaccine approach derives from the fact that influenza infection in humans leads to a series of complex interactions between the host immune system and the virus as the host endeavours to clear the infection [Borrow and Shaw, 1998]. Strain specific vaccine antibodies to the HA and NA antigens provide sterilising immunity by preventing virus entry into cells [Yewdell et al., 1979]. Whilst generally not sufficient to prevent infection, $CD8^+$ CTLs play a key role in viral clearance, promoting survival and recovery from severe infection with novel influenza strains [Wang et al., 2015] and accounting for asymptomatic seasonal influenza infections [Hayward et al., 2015]. Following influenza infection and CTL priming, primary CTL effector are recruited to the infected lung where they recognise antigenic peptides presented by major histocompatibility complex class I (MHC1) molecules [Kelso, 2012]. Following pMHC1 recognition on virus infected cells cytotoxic $CD8^+$ T cells kill these cells and secrete cytokines that result in viral clearance. Following influenza elimination some of the primary CTLs persist as long lasting memory cells that can be reactivated to mount a more rapid and powerful response, resulting in enhanced control during influenza re-infection [Boon et al., 2002b; Turner S, 2013].

Infection with one subtype of influenza A may result in protection against subsequent infections with heterosubtypic influenza A [Brown and Kelso, 2009; Zhong et al., 2010]. Studies have shown that prior influenza virus infections can induce protective heterosubtypic immunity [Hayward et al., 2015; Kreijtz et al., 2011; Sridhar et al., 2013; Wang et al., 2015; Wilkinson et al., 2012]. This cross-protective immunity is at least in part attributable to the $CD8^+$ T cell response which targets CTL epitopes derived from the more conserved internal proteins such as the NP and the matrix protein 1(M1) [Zhong et al., 2010]. While less variable than the HA and NA, these internal proteins still

undergo a degree of evolutionary change resulting in the emergence of novel CD8+ CTL epitopes among circulating influenza A strains [Boon et al., 2002b; Voeten et al., 2000].

Serologic studies provide helpful insights into likely antibody cross-protection against emerging influenza viruses [Grills et al., 2010; McVernon et al., 2010], but prediction of the likely prevalence of CTL cross-protection in the human population is challenging and is compounded by the fact that different age cohorts will have been exposed to a range of influenza variants over the course of a lifetime. Additionally diversity of MHC class I haplotypes, which determines the antigenic peptides presented in an individual and heavily influences CTL immunodominance [Boon et al., 2004] further complicates the study of key immunogenic peptides determining the profile of population immunity.

This study provides insight into the extent of diversification within the immunogenic NP peptides of H1N1, H2N2 and H3N2 viruses isolated from humans over the past century. The reference peptides against which these historical datasets are compared are derived from an H1N2 virus that circulated in the human population from 2000-2003. This virus was a reassortant of contemporary circulating H1N1 and H3N2 viruses, and was the first H1N2 virus in the reference collection observed to have circulated more than sporadically among humans [Barr et al., 2003]. The H1N2 strain infected individuals across the age spectrum, affecting children, young adults and the elderly, and there was no association with severe illness [Ellis et al., 2003; Komadina et al., 2014]. Its disappearance in 2003 followed an intra-subtype reassortment event between H3N2 viruses that occurred in 2002 [Holmes et al., 2005]. As the HA and NA of the H1N2 virus was both genetically and antigenically related to the circulating H1N1 and H3N2 viruses and a component of the vaccine at the time [Komadina et al., 2014] we hypothesised that relative novelty of CTL epitopes may have in part explained the success of the H1N2 virus.

Methods

Conservation analysis:

As not all epitope segments are known, here to ensure consistency we refer to both the longer immunogenic regions containing known minimal peptides as well as the immunogenic peptides themselves. A total of 46 H1N2 NP proteins of influenza viruses circulating from 2000-2003 were sourced from the Global Initiative on Sharing All Influenza Data (GISAID, www.gisaid.org)EpiFlu™ database to determine a consensus

sequence with 44 of the H1N2 NP proteins using Geneious V6.1.5 (www.geneious.com). A total of 73 H1N2 NP gene segments corresponding to experimentally defined class-1 restricted antigenic regions (www.iedb.org) were compared in 5 year blocks to a historical data set containing H1N1, H2N2, and H3N2 NP viral sequences circulating from 1918-2003. The 73 mapped H1N2 NP peptide sequences are shown in Supplementary Data, S1.

NP protein sequences for H1N1, H2N2 and H3N2 viruses from 1918-2003 were downloaded from the EpiFlu™ database with the subtype and isolate name for each sequence included in the fasta sequence header.

Data Preparation: Data were prepared using the alignment tool in Geneious V6.1.5 (www.geneious.com). Laboratory generated viruses and duplicate sequences with multiple passage history were discarded to minimise mutations in the regions of interest due to adaptation. Sequences which did not span the full range of peptides were also discarded. The H1N1, H2N2 and H3N2 sequences were merged into one data set giving a final test data set consisting of n=2207 sequences (Table 1). The data were trimmed to start from the first Methionine of the NP gene. A unique sequential ID number was inserted into the fasta header using an Excel macro (www.microsoft.com) in order to maintain a consistent, sequential order when tabulating the peptides of the test sequences (i.e.>1/H3/A/Sydney/5/1997).

Peptide Sequence Analysis: Each of the 73 peptide sequences was compared against the reference H1N2 sequence in 5-year blocks from 1933-2003. Only one NP sequence was available prior to 1933. The number of sequences and percentage of viruses with 100% match to the reference data were reported by subtype. The distribution of variants that did not match the reference data was also reported. Data were analysed using STATA V12.1 (www.stata.com).

Peptide diversity analysis: For the test data set of isolates the number of amino acid changes occurring in each peptide was calculated using an excel macro which provided output of each of the different sequence variations, the raw number of variants and percentage of each variant per peptide. Variations per peptide totalling ≤2 variants were not considered significant due to possible sequencing errors.

Results

The total number of influenza type A NP viral sequences used for the historical data set covering the time period of the study, 1918-2003 was 2207, Table 1. These consisted of

408 H1N1 viruses of which 29 circulated from 1918-1957 and the remainder post the re-introduction of the H1N1 viruses into the human population in 1977. In addition, there were 83 H2N2 and 1,716 H3N2 viral sequences.

H1N2 reference data: Of the 46 NP protein sequences sourced from GISAID, two sequences were discarded, as they were thought to be from an earlier reassortment event between H3N2 and H1N1 viruses, which did not persist [Komadina et al., 2014]. Of the remaining 44 sequences, 29 consisted of full length sequences and the remaining 15 were partial sequences. The 15 partial NP protein sequences were identical to the corresponding sections of the full length sequences. A consensus H1N2 NP sequence was generated using the full length and partial protein sequences, with the 73 H1N2 peptide sequences derived from this consensus sequence.

CTL antigenic region conservation across influenza viruses circulating between 1918-2003: Comparison of the peptides in H1N1, H2N2 and H3N2 viruses against the 73 reference H1N2 peptides showed that 11 of the immunogenic peptides were highly conserved across all 3 subtypes (Table 2) with 5 further of these also exhibiting a high degree (>95%) of conservation in the circulating viruses over the years of interest, 1918-2003. (Figure 1(a), Figure 2, pattern A). The remaining 57 peptides exhibited differing degrees of variation with 48 peptides ranging from 2 to 3 variants per peptide and 9 peptides with 4-10 variants per peptide with these latter peptides classed as highly variable. For most peptides, two major variants comprised more than 98% of their sequence. Approximately one third of peptides had 3-4 major variants. For 5 peptides, (3 overlapping NP_{89-111}, 2 overlapping $NP_{188-198}$) the peptide with a 100% match to the H1N2 sequence was the least frequently observed variant Figure 1(b).

Further analysis identified 15 antigenic peptides which were not detected in H1N1 or H2N2 subtype viruses (Figure 1(a)).

Temporal circulation of peptides: Mapping the circulation of antigenic peptides of interest over the study period, 1918-2003, shows a variety of temporal patterns. By visual inspection of the graphs, peptides with similar temporal sequences of circulation, similar emergence of variants or persistence in circulation were assigned pattern types (Figure 2). No statistical testing has been undertaken to differentiate these patterns. The full spectrum of temporal circulation patterns are shown in Supplementary Figure 2. Immunogenic peptides, which were conserved in their sequence, were also found to circulate in almost all viruses and in all three subtypes during the period of the study since the introduction of the type A H1N1 subtype in 1918

The emergence of some novel peptides was temporally associated with emergence of known pandemic strains (Figure 2). Three overlapping peptides found in $NP_{209-234}$ emerged with occurrence of the H2N2 pandemic viruses in 1957, pattern G. Four overlapping peptides in $NP_{397-416}$ region, which circulated in the early stages of the 1918 H1N1 pandemic ceased to circulate for some years, re-emerging with the advent of the H2N2 viruses, pattern D. Two other overlapping peptides in NP_{89-101} fleetingly observed re-emerged with H3N2 in 1968 but circulated in only 20% of viruses during the study period, pattern C. Other novel peptides that emerged in 1968 included $NP_{458-466}$ and 9 overlapping peptides found in $NP_{335-352}$, patterns I and H. A further 2 peptides $NP_{413-422}$ and $NP_{418-426}$ emerged with the re-introduction of the H1N1 viruses in 1977, pattern J.

Novel peptides were also seen to emerge within strains of a given subtype in the absence of antigenic shift. A major change in H1N1 viruses from 1937 saw the emergence of a number of overlapping novel peptides (6 peptides in $NP_{139-156}$) that remained fixed in both the H2N2 and H3N2 viruses, but by 2003 circulated in only about 10% of viruses, pattern E. A further two overlapping peptides emerged in 1947, $NP_{169-186}$, pattern F. These peptides circulated briefly before disappearing, re-emerging again in 1977 to become fixed by 2003. A number of novel peptides unique to H3N2 emerged during 1993-94 and rapidly became fixed in the H3N2 viruses. These peptides occurred in two regions of the NP gene, NP_{65-74} and 10 overlapping peptides in $NP_{373-396}$, pattern K.

The nine peptides previously described as immunodominant showed no apparent difference in the level of sequence variation compared with other peptides (Figure 3). Three of these peptides were observed from 1918 onwards (one falling to 40% prevalence in 1983) and were contained in all circulating viruses by 2003 [Boon et al., 2006; Grant et al., 2013; Wu et al., 2011]. Of another three other peptides circulating in all viruses by 2003, two emerged in 1968 (at which time prevalence was 20%) and a third ($NP_{383-391}$) was first noted in 50% of viruses in 1993. The three remaining peptides became less prevalent over time. $NP_{140-150}$ decreased from 100% during 1948-1998 to ≤10% by 2003, $NP_{404-413}$ was present in 1918 but disappeared during 1933-1953 re-emerging in 1993 but was only present in 60% of viruses by 2003. $NP_{418-426}$ emerged around 1978 in 30% of viruses, peaking at 100% of viruses round 1993 before decreasing to 60% of viruses by 2003. The rise of some novel peptides in this class coincided with major shift events in 1957 and 1968 or with the reintroduction of the H1N1 viruses in 1977. One of these peptides emerged in 1993 in H3N2 viruses and rapidly became fixed (Figure 3).

Discussion

The immunogenic NP peptide sequences of the H1N2 virus studied were identified and related to those observed from H1N1, H2N2 and H3N2 viruses of the past century. These peptides ranged from the highly conserved (15%) to highly variable (12%). Although no unique NP peptide sequences which were exclusive to the H1N2 virus were noted, the virus had however inherited two novel overlapping peptides $NP_{380-391}$ from a H3N2 variant which emerged during the 1993 – 1994 northern hemisphere winter.

Other studies have generally, either compared novel epitopes against small numbers of selected historical viruses, compared peptides in large numbers of viruses without assessing temporal variation or documented changes in a single epitope over time [Boon et al., 2004; Quinones-Parra et al., 2014; Rimmelzwaan et al., 2004b]. In the present study we extend a comprehensive approach by including all available viral NP sequences from 1918 until the disappearance of the H1N2 in 2003. The size and temporal distribution of this dataset allowed the first detailed evaluation and description of variable conservation of CTL antigenic peptides occurring within and across subtypes to the best of our knowledge.

The study had some unavoidable limitations of which one was the low number of NP sequences available from early in the 20th century, due to the lack of available influenza samples from that time period. Furthermore, not all human HLA-1restricted immunogenic or immunodominant peptides have been identified and only those previously described could be evaluated. A further limitation is that functional studies have only been carried out on a small number of peptides to provide understanding of the functional importance of observed sequence variants for immune escape.

Eleven peptides first noted in the NP of the 1918 H1N1 virus did not change throughout the period of the study and a further five had a high degree of conservation. This observed lack of variation over 85 years of the study may reflect functional constraints on mutation due to fitness costs [Berkhoff et al., 2005] or an absence of immune selection pressure [Voeten et al., 2000]. Overall the majority of peptides were relatively stable having one or two fixed variants that circulated in the viruses over time of the study.

Not surprisingly the introduction of novel subtypes, H2N2 (1957), H3N2 (1968) and the reintroduction of the H1N1 (1977), which co-circulated with the H3N2 viruses, was associated with emergence of novel peptides. Moreover, other emergent peptides were

observed in our dataset that accorded with epidemiological observations of major antigenic changes in circulating viruses. Whilst there is a paucity of sequence data available from the early 20th century, novel variants in 4 overlapping peptides, NP $_{139\text{-}154,}$ were first noted emerging in 1937 (Figure 2) in our dataset coinciding with published reports of influenza epidemics at the time[Hirst, 1943]. These emergent variants became fixed around 1947 coinciding with further antigenic changes in the virus which were associated with epidemics and the need to update influenza vaccine strains [Sigel et al., 1947]. These peptides persisted in a high proportion of all circulating viruses for a further fifty years, disappearing almost completely from circulating viruses with the emergence of an antigenically distinct H3N2 variant virus 1997-1998. This new variant gave rise to major epidemics in both the Northern and Southern hemispheres with large peaks in recorded deaths associated with H3N2 activity in the UK [Dedman et al., 1997]. These antigenic changes in the H3N2 virus necessitated a further update in the H3N2 component of the vaccine at that time [de Jong et al., 2000] .

Of interest were two overlapping peptides, $NP_{380\text{-}388}$ and $NP_{383\text{-}391,}$ which emerged in 1993 and were only found in the H3N2 and subsequently in the H1N2 viruses in 2000. The formerly observed variants of these peptides originally carried an arginine at position 384 from 1918-1989 and had been regarded as conserved until the emergence of an R384K mutation in 1989-1990. This mutation did not become fixed and was rapidly followed by an R384G mutation in 1993 which subsequently became stable, giving rise to the novel peptides observed in 1993 [Voeten et al., 2000]. This substitution occurred at the anchor residue for the HLA-B*0801 restricted $NP_{380\text{-}388}$ and the HLA-B*2705 restricted $NP_{383\text{-}391}$ overlapping epitopes, causing functional loss of the epitope for both HLA types [Berkhoff et al., 2004; Rimmelzwaan et al., 2004b]. Together these haplotypes are expressed in approximately 8% for HLA-B*2705 and HLA-B*0801 of the Caucasian population [Quinones-Parra et al., 2014; Voeten et al., 2000]. In vitro functional studies have identified the $NP_{383\text{-}391}$ peptide as immunodominant in the context of the HLA-B*2705 restricted response [Boon et al., 2002a]. The loss of this immunodominant peptide reduced the CTL response in vitro, leading to speculation that HLA-B*2705-positive individuals may be vulnerable to infection with viruses carrying this mutation [Berkhoff et al., 2004] and this may have contributed to the success of the H1N2 virus. This change furthermore appeared harmful to viral fitness and was tolerated only due to two further compensatory co-mutations in the NP [Rimmelzwaan et al., 2004a; Rimmelzwaan et al., 2005] This peptide is the only one noted which

produced an escape mutant in a previously conserved peptide that subsequently became fixed [Grant et al., 2014].

While most peptides were relatively conserved, 20% displayed a higher degree of variation with 12% particularly variable peptides displaying up to 10 variants each. Of the hyper-variable peptides, $NP_{418-426}$, restricted by HLA-B35+ is best characterised. While originally hypothesised to be under immune selection pressure, all of the observed variants still bind strongly to the HLA B35 and are recognised with high functional activity by CTLs [Derby et al., 2001; Gog et al., 2003].

Based on our screening parameters for variability, which required sequence identity to infer that there was no change, there were no obvious differences in variability of peptides classically described as immunodominant [Boon et al., 2006; Grant et al., 2013; Wu et al., 2011] compared with those that were not. Further evaluation of the degree of difference required to produce functionally distinct variants is required to assert with confidence that the strength of immune selection is not the main driver of diversity.

The H1N2 virus emerged not long after the 1997 intra-subtype reassortment event in the H3N2 viruses. The inter-subtype H1N2 reassortant virus had inherited seven of the H3N2 genes, meaning that any evolutionary fitness advantage gained by the 1997 H3N2 viruses were most likely conferred on the H1N2 virus. In 2002 another intra-subtype reassortment event occurred between the differing H3N2 lineages once again necessitating a change in the H3N2 vaccine due to loss of efficacy [Schultz-Cherry and Jones]. The new H3N2 viruses appeared to have gained a fitness advantage over the older H3N2 viruses. As the H1N2 virus carried the older constellation of H3N2 genes any evolutionary advantage they previously had which allowed these viruses to co-circulate for a period appears to have been lost and the H1N2 virus ceased to circulate in 2003.

Our comprehensive approach of comparing all the NP immunogenic regions in H1N2 virus against all previously circulating viruses in the humans has provided new insights into likely determinants of the virus' success. This approach has proven useful in examining emergent influenza viruses in the human population and in determining the likely extent of pre-existing CTL cross protection. Moreover, such studies inform on the temporal evolution of CTL antigenic regions and aid in selection of highly conserved peptides to be considered as universal influenza vaccine candidates.

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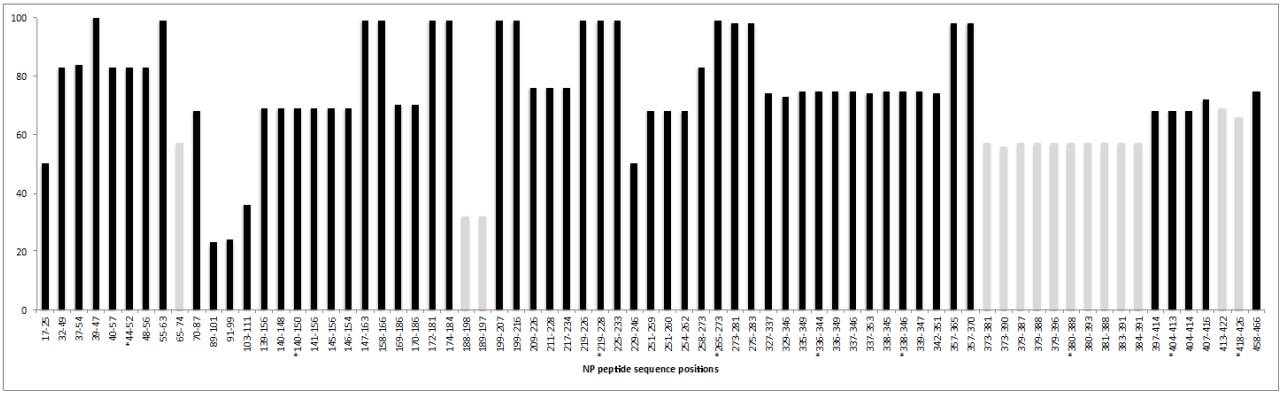
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Figures & Tables

Figure 1

(a)



(b)

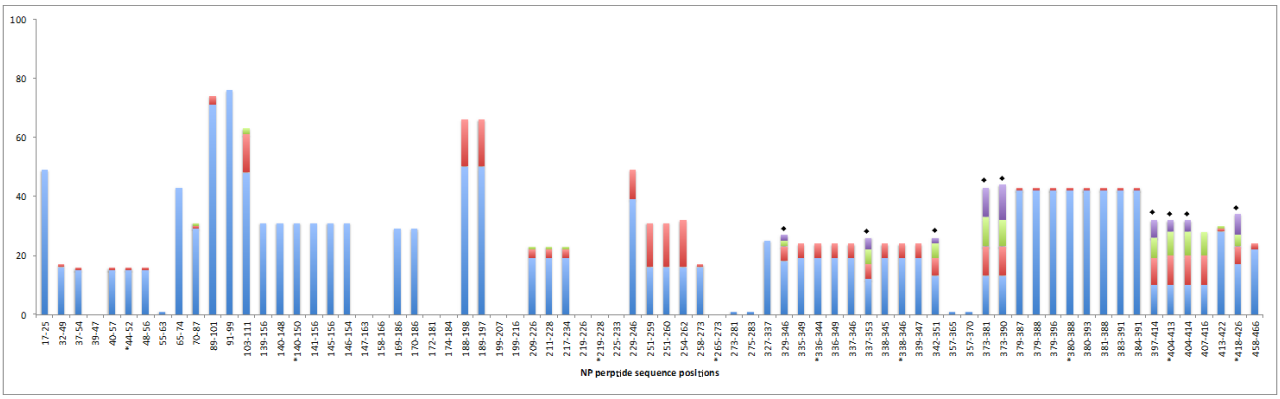


Figure 1: Summary of circulation of H1N2 NP peptides found in H1N1, H2N2 and H3N2 viruses during 1918-2003 and the variability occurring in the immunogenic peptides. Asterisks indicate peptides previously shown to be immunodominant in humans. (a) Specifies the percentage of peptides with a 100% match to the H1N2 peptides. The black bars indicate that these peptides circulated in all three subtypes and the grey bars indicate that these peptides circulated only in the H3N2 viruses. (b) Each colour represents one uniquely observed variant with the exception of the purple bar which indicates the highly variable 4 to 10 variants, with the ♦ symbol highlighting these hypervariable peptides.

Table 1:

Subtype	1918-1932	1933-1937	1938-1942	1943-1947	1948-1952	1953-1957	1958-1962	1963-1967	1968-1972	1973-1977	1978-1982	1983-1987	1988-1992	1993-1997	1998-2002	2003	Totals
H1N1	1	7	2	7	9	3	-	-	-	6	23	62	7	56	205	20	**408**
H2N2	-	-	-	-	-	16	22	39	6	-	-	-	-	-	-	-	**83**
H3N2	-	-	-	-	-	-	-	-	87	58	21	41	118	383	627	371	**1716**
Totals	1	7	2	7	9	19	22	39	93	64	54	103	125	439	832	391	**2207**

Table 1: The number of NP sequences for influenza type A H1N1, H2N2 and H3N2 subtypes available in 5-year groups from 1933-2003.

Table 2 Conserved NP immunogenic peptides in the H1N1, H2N2, H3N2 and H1N2 human viruses.

Immunogenic peptide	Sequence
NP 39-47	FYIQMCTEL
NP 55-63	RLIQNSLTI
NP 147-163	TYQRTRALVRTGMDPRM
NP 158-166	GMDPRMCSL
NP 172-181	LPRRSGAAGA
NP 174-184	RRSGAAGAAVK
NP 199-207	RGINDRNFW
NP 199-216	RGINDRNFWGENGRKTR
NP 219-226	YERMCNIL
NP 219-228	YERMCNILKG
NP 225-233	ILKGKFQTA
NP 265-273	ILRGSVAHK

Figure 2.

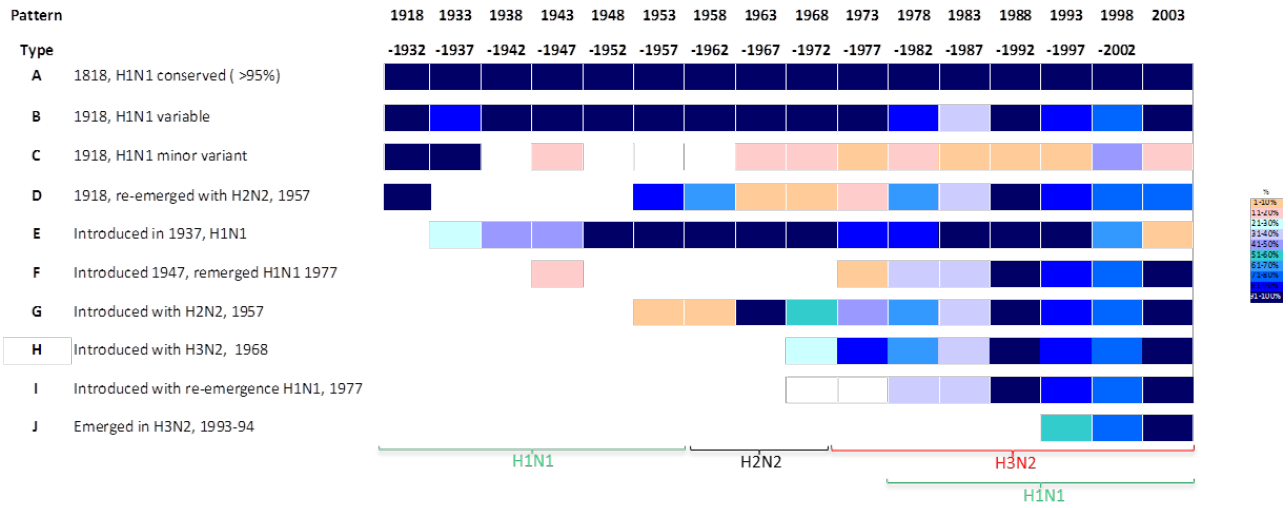


Figure 2: Example of the diversity of pattern types in the circulation of peptides between 1918 and 2003 among H1N1, H2N2 and H3N2 viruses. The proportion of strains in each five year block expressing the percentage of peptides with a 100% match to the reference H1N2 subtype is shown. The period of circulation of each subtype during the study is included on the x-axis for reference.

Figure 3

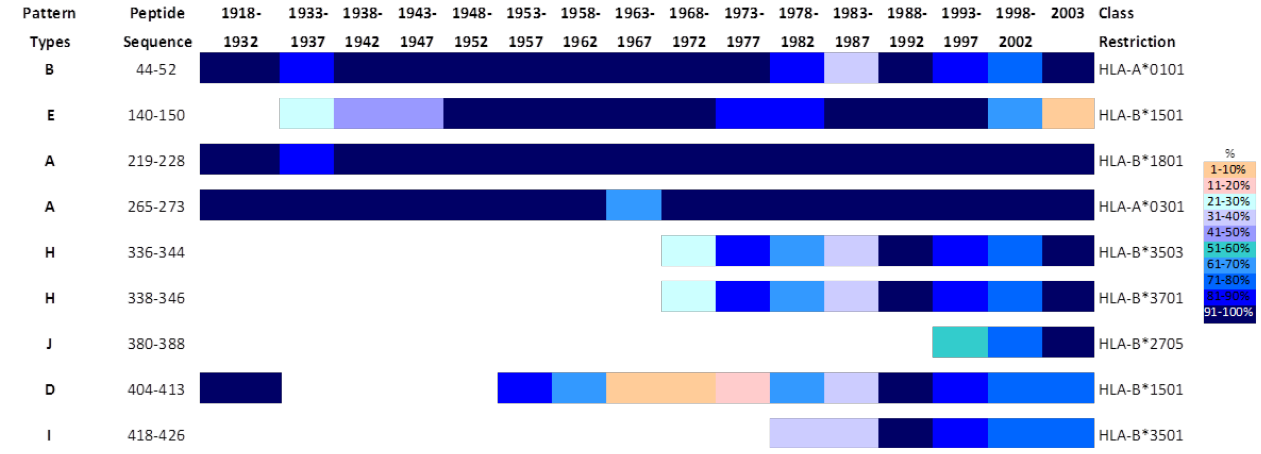


Figure 3: Circulation patterns of immunogenic peptides described as immunodominant and the HLA restriction types associated with the peptides [Boon et al., 2006; Grant et al., 2013; Wu et al., 2011] . Patterns C, F and G (Figure 2) were not noted amongst the immunodominant peptides.

Supplementary Data

S1: Positions and sequences of the H1N2 immunogenic peptides

	Position	Sequence
1	17-25	GDRQNATEI
2	32-49	MIDGIGRFYIQMCTELKL
3	37-54	GRFYIQMCTELKLSDYEG
4	39-47	FYIQMCTEL
5	40-57	YIQMCTELKLSDYEGRLI
6	44-52	CTELKLSDY
7	48-56	KLSDYEGRL
8	55-63	RLIQNSLTI
9	65-74	KMVLSAFDER
10	70-87	AFDERRNRYLEEHPSAGK
11	89-101	PKKTGGPIYRRVD
12	91-99	KTGGPIYRR
13	103-111	KWMRELVLY
14	139-156	WHSNLNDTTYQRTRALVR
15	140-148	HSNLNDTTY
16	140-150	HSNLNDTTYQR
17	141-156	SNLNDTTYQRTRALVR
18	145-156	DTTYQRTRALVR
19	146-154	TTYQRTRAL
20	147-163	TYQRTRALVRTGMDPRM
21	158-166	GMDPRMCSL
22	169-186	GSTLPRRSGAAGAAVKGI
23	170-186	STLPRRSGAAGAAVKGI
24	172-181	LPRRSGAAGA
25	174-184	RRSGAAGAAVK
26	188-198	TMVMELIRMVK
27	189-197	MVMELIRMV
28	199-207	RGINDRNFW
29	199-216	RGINDRNFWRGENGRKTR
30	209-226	GENGRKTRSAYERMCNIL
31	211-228	NGRKTRSAYERMCNILKG
32	217-234	SAYERMCNILKGKFQTAA
33	219-226	YERMCNIL
34	219-228	YERMCNILKG
35	225-233	ILKGKFQTA
36	229-246	KFQTAAQRAMVDQVRESR
37	251-259	AEIEDLIFL
38	251-260	AEIEDLIFLA
39	254-262	EDLIFLARS
40	258-273	FLARSALILRGSVAHK
41	265-273	ILRGSVAHK
42	273-281	KSCLPACVY
43	275-283	CLPACVYGP
44	327-337	QLVWMACHSAA
45	329-346	VWMACHSAAFEDLRLLSF
46	335-349	SAAFEDLRLLSFIRG
47	336-344	AAFEDLRLL
48	336-349	AAFEDLRLLSFIRG
49	337-346	AFEDLRLLSF
50	337-353	AFEDLRLLSFIRGTKVS
51	338-345	FEDLRLLS
52	338-346	FEDLRLLSF
53	339-347	EDLRLLSFI
54	342-351	RLLSFIRGTK
55	357-365	KLSTRGVQI
56	357-370	KLSTRGVQIASNEN
57	373-381	NMGSSTLEL
58	373-390	NMGSSTLELRSGYWAIRT
59	379-387	LELRSGYWA
60	379-388	LELRSGYWAI
61	379-396	LELRSGYWAIRTRSGGNT
62	380-388	ELRSGYWAI
63	380-393	ELRSGYWAIRTRSG
64	381-388	LRSGYWAI
65	383-391	SGYWAIRTR
66	384-391	GYWAIRTR
67	397-414	NQQRASAGQISVQPTFSV
68	404-413	GQISVQPTFS
69	404-414	GQISVQPTFSV
70	407-416	SVQPTFSVQR
71	413-422	SVQRNLPFEK
72	418-426	LPFEKSTVM
73	458-466	FRGRGVFEL

S2 Full plots of temporal distribution with periods of subtypes circulation noted below.

