Marked Rise in Replikin Counts in H5N1 Influenza Virus Localized to Lethality Gene p B1.

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Abstract: Virus outbreaks have been found to be related to the concentration of a new class of genomic peptides, Replikins¹. The eight genes of H5N1 influenza virus were analyzed for the distribution of Replikin Counts (number Replikins /100 amino acids) in 2,441 sequences from birds and humans. An increase (p<0.001) occurred from 2004 to August 2011 in one gene, p B1.

Replikins Count

Legend for Figure: Eight Groups of Replikin Counts, one Group [ ] for each gene: [NS1], [Matrix], [pA], [Hemagglutinin], [p B1], [Neuraminidase], [p B2], [Nucleoprotein]. Two Subgroups are shown for each gene: Counts on virus isolates from birds [B], Counts on virus isolates from humans [H]. Each Subgroup contains 8 annual Replikin Counts, one for each year available from 2004 to 2011 (no data on PubMed in some years).

Total Number of sequences analyzed for Replikins= 2,441
Number of sequences in each subgroup:
[10 /7] [277 / 466] [153/ 62] [209/ 425] [191/ 316] [194 / 322] [140 /75 ] [142 /212]

In H1N1, the Replikin Count analyses of the hemagglutinin (‘Infectivity’) gene predicted the 2009 pandemic one year in advance. This analysis then was repeated every few days to monitor ‘real-time’ the course of the pandemic¹. In H5N1, the potential for high lethality of H5N1 was predicted for Indonesia in 2006 by a rising H5N1 Replikin Count, then realized in 2007. In H5N1 currently, the continuing increase in the p B1 Replikin Count to record highs in August, 2011, and the current outbreaks of high lethality H5N1 in Egypt and Asia, are therefore of concern. The increasing Replikins in H1N1 hemagglutinin, together with the localization of the H5N1 Replikin Count increase to p B1, has led to the development of a completely synthetic Replikins pan-influenza vaccine effective against H5N1².
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### Introduction

Replikins are the only conserved structures of infectious organisms described to date which correlate quantitatively and temporally with epidemic outbreaks, course, and lethality, and permit early or advance warning of such outbreaks. Replikins are genomic structures defined by the algorithm: peptides 7 to 50 amino acids long, containing two or more lysines, six to ten amino acids apart, at least one histidine, and a lysine concentration of 6% or more\(^3\). Replikins have been counted and their spread over the gene’s surface has been visualized in the hemagglutinin gene by X-ray diffraction in pre-pandemic and pandemic periods\(^1\).

### Methods

All H5N1 genomic sequences in Pubmed were analyzed by software based on the authors’ algorithm\(^4\). Replikin peptides were first identified and then counted in each genomic sequence (Replikin Count = number of replikins per 100 amino acids). For each group of specimens’ replikins, the mean and standard deviation of the mean (SD) were calculated and compared between 2004 and August 2011. The terms ‘increase’ and ‘decrease’ of Replikin Counts were used only when the p level was less than 0.001. Counts in H1N1, H5N1 and other influenza strains were each monitored separately. All sequence data submitted to Pubmed by laboratories throughout the world relevant to the keyword subjects of the inquiry were analysed. The number of sequences published on Pubmed for each inquiry are indicated in the Figure. Some years had no sequences published for some genes. During outbreaks, Replikin Counts were compared to Counts for the same strain in non-outbreak time periods. Replikin genes were isolated \textit{in silico} by scanning and identifying those areas of the virus genome which had the highest concentration of Replikins. When a change was found to be associated with high infectivity (morbidity over the time period, CDC and WHO epidemiological data), the high Count area was named Replikin Infectivity Gene; Infectivity Genes were found in the hemagglutinin area. Similarly, areas in the genome were sought which were associated with high Replikin Counts and high mortality rates; the high Count area then was named Replikin Lethality Gene. In the present study, the Replikin Lethality Gene was found specifically in the p B1 area.

### Results and Discussion

In contrast to the rising Replikin Counts in one gene, p B1, the constancy in the other seven out of eight of the influenza genes of both the mean and the standard deviation of the Replikin Counts, over a seven and one half year period, is notable. Confidence is increased in the reliability of the software methods used by the presence of this constancy over years. Further, controls are thereby provided for the evaluation of changes in that constancy when they occur. An independent statistical analysis for H1N1\(^1\) of the significance of the changes in Replikin Counts is relevant as precedent to evaluating both
the constancy and changes observed in H5N1 in the present study. Rising replikin concentration in H1N1 from 2006 to 2008, predicted one year in advance the H1N1 outbreak of 2009. A highly significant increased concentration of virus replikins was found prospectively before the H1N1 2009 pandemic (12,806 sequences) (in the hemagglutinin gene (N=8,046), p values by t-test = 1/10^{10}, by linear regression = 1/10^{24} and 1/10^{29}, by Spearman correlation < 2/10^{16}, by Wilcoxon rank sum<1/10^{16} by multiple regression adjusting for correlation between consecutive years = 2/10^{22}.

Mean Replikin Counts in six of the eight genes of H5N1 are seen in the Figure to be under 5, as in earlier evidence found for H1N1 genes in non-outbreak ‘resting’ periods. In all genes with mean Replikin Counts under 5, the Figure shows that the SD is small, reflecting the restricted range of the virus population. Although the hemagglutinin gene, showed a recent significant increase in the means in birds (non-significant in humans), the standard deviation of the mean (SD) increases shown in the Figure indicate that ‘scout viruses’ with increased Replikin Counts have appeared among the virus population, possibly signalling a coming increase in H5N1 infectivity.

Surprisingly, while a major reservoir and vector for H5N1 is thought to be in birds, as seen in the Figure, the ‘preferred’ host in terms of recent increases in Replikin Count appears to be humans.

**H5N1 Outbreaks and Lethality**

In H5N1, increased Replikin Counts predicted the lethal outbreaks of H5N1 between 1997 and 2010. H5N1 is very different from H1N1 in terms of its infectivity and lethality. H5N1’s infectivity is lower and lethality higher than H1N1. During the worst recorded period of H1N1, the 1918 pandemic, the mortality rate was estimated to be only 2.5%, whereas the human mortality rate for H5N1 has been reported by WHO to be as high as 82%. From specimens of 1957, when H5N1 sequences were first reported, until 1996, the mean Replikin Count did not exceed 4. A slight but statistically significant increase in H5N1 Lethality Gene Replikin Count occurred in 1996, in advance of the outbreak in Hong Kong in 1997. In 1996, an increase in Replikin Count preceded the 2007 Hong Kong outbreak in which 30 human cases occurred with a mortality rate of 27%. The Count increased between 2005 and 2008 (Figure) with subsequent animal and human outbreaks in Asia and Egypt in 2007-2010 (WHO).

**Prediction of Geographic Location of H5N1 (Avian flu) Outbreak in Indonesia**

Instead of comparing neighboring genes, neighboring countries were compared for the Replikin Counts of H5N1 scout infections in humans over several years. In the replikin prediction of 2005-2006, Indonesia was predicted to be the country that would be worst affected in terms of increased human mortality rate. Following the replikin prediction, 277 human H5N1 cases were reported and the human mortality rate increased in Indonesia from 40% to 82% (WHO).
Concurrent H5N1 and H1N1 Build-Up in 2011

H5N1.
Because of the increase in H5N1’s Replikin Count in birds from 2002 to 2008 (Figure), and the increased Counts in H5N1’s proposed precursor H9N2 in chickens\(^8\), the authors issued a warning in January 2009 that H5N1 outbreaks would surge\(^9\). By January of 2010, H5N1 outbreaks occurred in birds and chickens in 63 countries\(^10\). Human cases appeared monthly, most prominently in Egypt, where there were 106 WHO confirmed human cases and 32 deaths (mortality rate 30.2%) as of March 16, 2010. Also in March, 2011, the case-fatality rate was reported by CIDRAP to be 34%, versus 60% for other countries with human cases. Now, as of June and July, 2011 respectively, the cumulative mortality rate is reported by WHO to have risen since 2006 to 34.7%; and the current mortality rate reported by the Egyptian government is 38.7%. Globally, as of 2010, the H5N1 hemagglutinin Infectivity Gene Replikin Count had reached its highest level in humans since 1998, 4.6+/−4.3\(^1\).

H1N1.
The H1N1 Infectivity Gene Replikin Count, never returned to pre-pandemic levels in 2010; from the outbreak in April 2009 its peak Replikin Count persisted at approximately 10 through 2010, then peaked globally in January 2011 at 13.5+/−4.2. In Mexico, 3/21 specimens in the first four months of 2011 had record high levels of 16.7. The failure of the Replikin Infectivity gene to return to pre-2009 outbreak levels two years later is in marked contrast to the SARS Replikin Count which promptly returned to pre-outbreak Replikin levels in approximately 9 months\(^1\) to signal the end of the SARS outbreak. The present data therefore strongly suggests that the H1N1 pandemic of 2009 is continuing to develop. While there were insufficient H1N1 p B1 sequences submitted to PubMed from Mexico as of August, 2011, the global H1N1 Lethality Gene Replikin Count level in humans, which had decreased at the end of 2010 to its pre-pandemic level of 2.0+/−0.2, was again increased to 5.6+/−4.9 as of August 2011, higher than the range that it was in before the 2009 H1N1 pandemic\(^1\).

Conclusions

The elevation of the concentration of both the H1N1 Infectivity and Lethality Genes, is invariably (in 18/18 predictions) followed by clinical outbreaks\(^13\). The concurrent combined activation of both the Infectivity and Lethality Genes of H5N1 as well in 2011 therefore is of concern\(^1\). The 2011 outbreaks have begun for both H1N1 and H5N1. Again as for H1N1 in 2008, in Mexico, initial ‘scout’ virus outbreaks of H1N1 have occurred in 56 cases with Replikin Counts of the Infectivity Gene up to a record 16.7 and a human mortality rate of 10.7%\(^13,14\). Outbreaks of H5N1 in Egypt have begun with a current cumulative mortality rate of from 34.7% (3.4) to 37.8%\(^5,6,12\). A structural build up of virus Replikins, in both H1N1 and H5N1, consistent with that observed in advance of the last influenza pandemic of 2009\(^1\), therefore appears to be in progress towards another pandemic of one or both strains.
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