**Genome Replikin Count™ Predicts Increased Infectivity/Lethality of Viruses**

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**Figure 1. Three-Dimensional Visualization of actual amino acids of virus H1N1 hemagglutinin before and during the 2009 H1N1 Pandemic** shows the increasing appearance of replikin structures on the surface of the hemagglutinin gene, and the encirclement of the sialic acid contact area of the virus, as the Replikin Counts increased from 3.2 to 10.1 during the Pandemic’s development.

Increasing replikin count correlates with expanding replikin surface area coverage in H1N1 haemagglutinin. Based on sequence alignment between a progression of H1N1 strains with increasing replikin count (3.2, 5.5, 10.1), relative replikin surface area is shown superimposed onto the 1918 H1N1 strain haemagglutinin (grey).

Replkins are in cyan. Contact points for the sialic acid host receptor are shown as orange spheres. Note at 3.2 there are no visible replikins around the sialic acid. At 5.5 the partial, and at 10.1 the almost complete encirclement of the sialic acid site by replikins. The maximal surface coverage is achieved at Replikin Count of 10.1; the surface at Replikin Count of 11.7 (not shown) is the same as that at 10.1.

This communication is one of four submitted together:

- Genome Replikin Count™ Predicts Increased Lethality of Resistant Tuberculosis
- Genome Replikin Count™ Predicts Increased Lethality of Malaria
- Genome Replikin Count™ Predicts Increased Lethality of Cancer
- Genome Replikin Count™ Predicts Increased Infectivity/Lethality of Virus
Legend for Figure 2. Surface representation of influenza hemagglutinin of the 1918 H1N1 pandemic virus showing replikin distribution (cyan) and CR6261 antibody (21,22) binding site. The antibody binds on the outer surface of the hemagglutinin with contact on and binding to amino acid residues (red) of replikins (cyan). All 18/18 amino acid contact and binding sites in HA2 for the antibody are parts of the additional replikins covering the surface in this area at the Replikin Count of 10.1 and higher, but not present at Replikin Counts of 3.2 and 5.5, earlier in the development of the pandemic (Figure 3a).
Figure-Annual Genomic Replikin Count Analysis of Foot and Mouth Disease Virus

| Replikin Count | (=number of Replikins per 100 amino acids) |
|----------------|-----------------------------------------------|
|                |                                                |

Replikin Count™ Cycling Observed in Viruses

The cycling of the Replikin Count and the concomitant association of infectivity/lethality observed for Plasmodium Falciparum (Figure 1, Genome Replikin Count™ Predicts Increased Lethality of Malaria) has also been observed in viruses, namely, the H1N1, H2N2, H3N2, H5N1, and H3N8 strains of influenza virus and in West Nile virus and Foot and Mouth Disease Virus. Thus Replikin cycles are observable in viruses as well as in Plasmidia. The Replikin concentration of West Nile Virus was found to increase annually through two distinct cycles as the virus expanded in the U.S.: the first from 2000 to 2003, and the second from 2004 to 2007 (p less than 0.001). Increases in the annual number of CDC reported human cases followed each of the virus Replikin concentration increases. Mortality rates are not reliable but follow morbidity at 5 to 30% (CDC). This is the first report that cyclic increases in virus replikin concentration, each apparently building on the last, can be a mechanism of virus expansion into a
The mosquito-born West Nile virus, which incubates between seasons, has been found to increase its concentration of replikins before the next annual step in the cycle of expansion. The demonstration of replikin cycles represents further 'proof of principle' on the relationship of replikins to virus epidemics and a new means of discerning the course of an epidemic. Previously, increases in the ReplikinsCount™ of the Replikins Peak Gene were found to precede and to predict outbreaks and lethality of two other viruses, influenza H5N1 and Taura Syndrome Virus, in two hosts, human and shrimp respectively. The conservation of specific replikin structures over many years, the detection of new replikins, and the ability to chemically synthesize replikin vaccines in 7 days, demonstrated for H5N1 and Taura Syndrome viruses, have now also been demonstrated for West Nile Virus.

Similar correlations also have been shown for Replikin concentrations and human mortality in an influenza H5N1 cycle between 1997 and 2007(1). Replikin Count analysis of Foot and Mouth Disease Virus (FMDV) have also indicated cycling and the Replikin Count of 2009, the highest ever recorded for FMDV, predicted the FMDV outbreaks of 2011-2012 (see accompanying communication Genome Replikin Count™ Predicts Increased Infectivity/Lethality in Viruses).

RELATION OF GENOMIC REPLIKIN COUNT TO MORTALITY OF H1N1 AND H5N1 INFLUENZA
'Replikin Peak Gene' of H5N1 Virus: Activity in Four Hosts:

**HOST LOCALIZATION**

- Goose: 01-02, 03, 04-05, 06
- Duck: 02-03, 04, 05-06
- Chicken: 02-03, 04, 05-06
- Human: 01-02, 03

**Localization of 'Replikin Peak Gene'**

- Series 2
- +/- SD
- Mean

Replikin Count per 100 amino acids
H5N1 RPGs of each country. The results are shown for the Replikin Count for all data available on PubMed each year 2003-2006. Low levels of Replikin count, below 4, were observed in each host group until 2005-2006, when human H5N1 increased in Asian countries. Human RPG activity was upregulated in 2005-2006 most prominently in Indonesia. Using this data, the authors predicted Indonesia would be the country most likely to first experience increased human mortality. The prediction was proven correct in 2007 when incidence of human morbidity and mortality in the Indonesian outbreak were exceptionally high and evidence of possible human to human transmission was observed. Changes in Replikin Count in the Replikin Peak Gene of the H5N1 isolates such as in Figure 6 allows for identification of those geographic areas in which the influenza virus strain is more virulent than other geographic areas. U.S. Appln. Ser. No. 12/010,027 (published).

**Figure 1. Three-Dimensional Visualization of actual amino acids of virus H1N1 hemagglutinin before and during the 2009 H1N1 Pandemic** shows the increasing appearance of replikin structures on the surface of the hemagglutinin gene, and the encirclement of the sialic acid contact area of the virus, as the Replikin Counts increased from 3.2 to 10.1 during the Pandemic’s development.
Figure 6 - The increased Replikin Counts in the RPG of H5N1 influenza virus (e.g., SEQ ID NO: 1684) may be directly correlated with human mortality. Supplement Figure 6 illustrates the relationship of Replikin Count of the Replikin Peak Gene in human H5N1 to percent human mortality between 2003 and 2007 in human cases of H5N1 infection. An increase in Replikin Count in the Replikin Peak Gene of H5N1 is observed to be quantitatively related to higher mortality in the host. The Replikin Peak Gene in human H5N1 is in the polymerase gene area, which has the highest concentration of continuous Replikin sequences in publicly available sequences of the H5N1 genome.
Marked Rise in Replikin Counts in H5N1 Influenza Virus Localized to Lethality Gene p B1.

Samuel Bogoch* and Elenore S. Bogoch**

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 Mean, black Count SD, red

See Figure

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

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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 more3. 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 periods1.
Methods

All H5N1 genomic sequences in Pubmed were analyzed by software based on the authors’
algorithm. 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
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 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 = 1/10130, by linear regression = 1/1024
and 1/1029, by Spearman correlation < 2/1016, by Wilcoxon rank sum<1/1016, by multiple
regression adjusting for correlation between consecutive years = 2/1022.

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’ 1 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 41. 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, the authors issued a warning in January 2009 that H5N1 outbreaks would surge. By January of 2010, H5N1 outbreaks occurred in birds and chickens in 63 countries. 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+/-.31.

**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+/-.2. In Mexico, 3/21 specimens in the first four months of 2011 had record high levels of 16.71. 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 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 pandemic1.

**Conclusions**

The elevation of the concentration of both the H1N1 Infectivity and Lethality Genes, is invariably (in 18/18 predictions) followed by clinical outbreaks1,3. The concurrent combined activation of both the Infectivity and Lethality Genes of H5N1 as well in 2011 therefore is of concern1. 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 20091, therefore appears to be in progress towards another pandemic of one or both strains.

**Acknowledgements**

We are grateful to Pubmed, and to its contributors, whose data were used extensively in these studies. Dr. S. Winston Bogoch, with the authors, designed the software used in this study1. Anne Bogoch Borsanyi was responsible for the preparation of research protocols and contributed to the writing of this manuscript. We are grateful to Professor Mark Jackwood, University of Georgia, for the independent testing of synthetic replikins TransFlu™ vaccine against H5N1 in chickens2. We are grateful to the United Kingdom Department of Trade and Investment for sponsoring academic seminars to introduce the technology of the Replikins Bioradar Global Surveillance System™15.

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In Figure 16, a correlation was established between human mortality and (1) mean concentration of Replikin sequences in the whole genome, (2) mean concentration of Replikin sequences in the polymerase gene, and (3) mean concentration of Replikin sequences in the Replikin Peak Gene (pB1 gene area) of H5N1 influenza strains. As Replikin concentration increased by these three measures, human mortality was observed to increase. However, while all three measures provided a correlation with human mortality, changes in the Replikin Count in the polymerase gene correlated more significantly with human mortality, and changes in the Replikin Count in the Replikin Peak Gene (pB1 gene area) of the H5N1 genome correlated still more significantly with human mortality. Figure 16 suggests, therefore, that identification of Replikin Peak Genes within viral genomes improves identification and prediction of virulence and mechanisms of virulence using Replikin concentration data.

Human Replikin Peak Gene (RPG) activity was upregulated in 2005-2006 most prominently in Indonesia. Using this data, The authors predicted Indonesia would be the country most likely to first experience increased human mortality. The prediction was proven correct in 2007 when incidence of human morbidity and mortality in the Indonesian outbreak were exceptionally high and evidence of possible human to human transmission was observed. Changes in Replikin
Count in the Replikin Peak Gene of the H5N1 isolates such as in Figure 6 allows for identification of those geographic areas in which the influenza virus strain is more virulent than other geographic areas. U.S. Appln. Ser. No. 12/010,027 (published).

**Increase in Replikin Count in 2002 in all Spike and Nucleocapsid Coronavirus Proteins Preceded the SARS Coronavirus Pandemic of 2003**

The pre-pandemic increase in both nucleocapsid and spike coronavirus proteins is in accord with, and might have served as a warning of, the finding that a coronavirus is responsible for the 2002-2003 first SARS pandemic (Table IX). Figure 6 depicts the results of part of the automated Replikin Analysis (see below) of nucleocapsid and spike coronavirus proteins for which the protein sequence is available on isolates collected from 1995 to early 2004. This software is
now available for similar replikin quantitative analysis of any protein. Each individual protein is represented by an accession number and is analyzed for the presence of replikins. Replikin peptides are detected in each sequence and each replikin can be traced for its presence or absence in each year for which the protein sequences are available. The Replikin Count (number of replikins per 100 amino acid) is automatically calculated. For each year, the mean (± standard deviation (S.D.)) Replikin Count per year is automatically calculated for all Replikin Counts that year. One or more proteins can be included in each analysis. This example of early warning of increasing replication, before an epidemic, of two particular proteins, in this case the nucleocapsid and spike proteins, in a particular virus group, in this case the coronaviruses, is comparable to the increase seen in strains of influenza virus preceding influenza epidemics and pandemics (Figures 1-5). It may be seen that the Replikin Count rose between 1995 to 2002, consistent with the SARS coronavirus pandemic, which emerged at the end of 2002 and has persisted into 2003. For example, the Replikin Counts are obtained as shown below for the coronavirus nucleocapsid protein replikins from 1995 to early 2004. Since sequence analyses are constantly occurring on both new and old isolates, and as can be seen below, in large numbers in epidemic years, the Replikins.

I. LABORATORY DEMONSTRATION OF RELATIONSHIP OF REPLIKIN COUNT TO PERCENT MORTALITY IN TAURA SYNDROME VIRUS INFECTION IN SHRIMP

To test further the relationship of Replikins to virulence, the relationship of Replikin count of shrimp viruses to mortality in shrimp was examined in a controlled situation. Based on the hypothesis that the Replikin count of a virus is related to virulence of the virus and the percent mortality of the host, as developed from the evidence on H5N1 virus infections in
humans, the authors tested whether it would be possible to predict solely from the Replikin Count of the amino acid sequence of the whole genome what the order of virulence would be of four strains of the virus. Taura syndrome shrimp virus (TSV), which kills most host shrimp within a few days of infection, was chosen to be studied. The amino acid sequences of four strains of taura syndrome virus (Belize, Thailand, Hawaii, and Venezuela) were analyzed with the FluForecast® software of REPLIKINS LLC, Boston, MA and the results held in confidence until the laboratory challenge experiments with the virus were completed, then compared with the percent mortality produced by each strain. U.S. Appln. Ser. No. 12/010,027 (published).

In the laboratory, there was a significant linear correlation between the mortality rates of the host shrimp challenged with each of the four virus strains and the mortality rates predicted earlier by only the Replikin counts of each strain. These data support the conclusion that virus Replikin peptide concentration, in addition to predicting virus outbreaks, relates quantitatively to host mortality rate and to the increase in virulence over time observed. U.S. Appln. Ser. No. 12/010,027 (published).

Replikin Analysis

Visual Replikin analysis was performed on the sequence information for the taura syndrome virus isolates from Belize, Thailand, Hawaii, and Venezuela by applying the algorithm defining Replikins with computer access to protein and genomic sequences freely available on PubMed or other public databases. The specific defining algorithm follows: a Replikin is a peptide sequence in a protein or genome, 7 to 50 amino acids long having a terminal lysine and a terminal lysine or histidine, containing at least 2 lysine groups 6 to 10 amino acids apart, at least 1 histidine group, and at least 6% lysine. Overlapping Replikins are common and are counted separately. The quantitative correlations with rapid replication and epidemics and lethality require all components of the algorithm to be in place for each Replikin. Thus for example, if the length and lysine requirements are present but there is no histidine present, the peptide is not a Replikin. Automated Replikin analysis was performed with the FluForecast® software service of Replikins Ltd., Boston, MA.

Identification of the Replikin Peak Gene

The Replikin count was used to identify that area of the genome which had the highest concentration of Replikins, and this area called the Replikin Peak Gene (RPG) area. The further two to eight-fold increase in the Replikin count of the RPG which occurred with outbreaks was further used to confirm the identity of this gene. The function of the gene was therefore used to identify it or isolate it “in silico”.

Nature Precedings : doi:10.1038/npre.2012.7144.1 : Posted 3 Apr 2012
Shrimp Virus Laboratory Methods

At the Aquaculture Pathology Laboratory, Department of Veterinary Science and Microbiology, University of Arizona, Tucson AZ, small juveniles of specific-pathogen-free *Litopenaeus vannamei* shrimp per tank, mean weight: 1.8 g, were fed minced TSV-infected tissues (infected separately with each of the 4 isolates originating from Belize, Thailand, Venezuela and Hawaii) for 3 days at 5% of their body weight. These shrimp were maintained with pelleted ration (Rangen 35%) for the following 12 days. Each challenge bioassay of a specific isolate was done in triplicate. During the bioassay period, all tanks were checked daily for dead or moribund shrimp. All mortalities were removed from the tank and frozen. One to three moribund shrimp from each isolate were preserved in Davidson’s AFA fixative and processed for routine histology to confirm viral infection. For each isolate, six moribund shrimp were collected during the acute phase infection and total RNA was extracted from their gill tissues with a High Pure RNA tissue kit (Roche). The extracted RNA was analyzed for the presence of TSV by real-time RT-PCR. All tanks were outfitted with an acclimated biological filter and aeration, and were covered with plastic to contain aerosols. The average salinity of the water was 23 ppt and the water temperature was 28°C. The challenge study was terminated after 15 days with live animals counted as survivors. U.S. Appln. Ser. No. 12/010,027 (published).
Supplement Figure 14 illustrates a correlation between cumulative survival of *Litopenaeus vannamei* shrimp challenged with four different taura syndrome virus isolates over 15 days (unless 100% mortality occurred prior to 15 days) and the Replikin concentration of Open Reading Frame 1 (ORF1) of each isolate. Translated amino acid sequences of ORF1 of the genome of individual isolates of TSV from Belize, Thailand, Hawaii and Venezuela were analyzed for Replikin Count. Replikin Count was determined to be 3.5 for the Belize isolate, 3.4 for the Thailand isolate, 3.3 for the Hawaii isolate and 3.0 for the Venezuela isolate. Graph A illustrates observed percent survival in three trials of shrimp challenged with the Belize isolate of TSV. In one trial, total mortality was observed on day 6. In the other trials, total mortality was observed on day 11. Graphs B, C and D illustrate observed percent survival of shrimp challenged with the Thailand isolate, the Hawaii isolate and the Venezuela isolate, respectively, each in three trials over 15 days. In the Thailand isolate, a mean of 80% percent mortality was observed on day 15. In the Hawaii isolate, a mean of 78.3% mortality was observed on day 15. In the Venezuela isolate, a mean of 58.3% mortality was observed on day 15. U.S. Appln. Ser. No. 12/010,027 (published).

Supplement Figure 15A illustrates a direct sequential correlation between Replikin Count in isolates of taura syndrome virus (TSV) collected from Belize, Thailand, Hawaii and Venezuela, respectively, and mean number of days to 50% mortality in *Litopenaeus vannamei* shrimp challenged with the respective TSV isolates beginning on day one through day three. Statistical differences between the Replikin concentration for each isolate are significant at a level of p<0.001. U.S. Appln. Ser. No. 12/010,027 (published).
Supplement Figure 15B illustrates a direct correlation between Replikin Count in isolates of taura syndrome virus (TSV) collected from Belize, Thailand, Hawaii and Venezuela, respectively, and mean cumulative survival of *Litopenaeus vannamei* shrimp at 15 days after challenge with the respective TSV isolate. Statistical differences between the Replikin concentrations for each isolate are significant at a level of p<0.001. U.S. Appln. Ser. No. 12/010,027 (published).

**Comparison of Virulence**

First mortality was seen on day 2 after exposure to TSV in all 4 isolates. For Belize isolate, most (83%) of shrimp died by day 4 and had a 0% survival at day 11 (Fig 14A, Table 26). For Thailand isolate, 63% mortalities occurred by day 4 and had 20% survivals at the end of 15-day bioassay (Fig 14B, Table 26). For Hawaii isolate, mortalities increased starting at day 2 and reached to a peak at day 5; the cumulative survival is 22% at the end (Fig 14C, Table 26). For Venezuela isolate, mortalities occurred slowly at days 2 and 3 with 22% of shrimp showed mortalities on day 4 and then mortalities were slowing down; there were 42% of shrimp survived in the end (Fig 14D, Table 26). The time period for reaching 50% mortality caused by TSV infection for the isolate of Belize, Thailand, Hawaii and Venezuela were 2.8, 3.5, 4.5 and 7 days, respectively (Table 26).

The data from Supplement Figure 14 is contained in Supplement Table 26 below. U.S. Appln. Ser. No. 12/010,027 (published).

**Supplement Table 26 – TSV Challenge**

| TSV isolate | GenBank no. (ORF1) | Survival (%) (Mean) | Day of 50% mortality |
|-------------|--------------------|---------------------|----------------------|
| Belize      | AAT81157           | 0                   | 2.8                  |
| Thailand    | AAY56363           | 20                  | 3.5                  |
| US-Hawaii   | AAK72220           | 22                  | 4.5                  |
| Venezuela   | ABB17263           | 42                  | 7.0*                 |

*High variation was observed in Venezuela’s triplicate tanks, thus the Day of 50% mortality was determined by Kaplan-Meier survival analysis with the Statistix 8 program.

The correlation of the virulence observed for each of the TSV isolates with the predicted virulence by Replikin Count alone are shown in Supplement Figure 15. Supplement Figure 15A provides data comparing Replikin Counts of the four isolates with the mean day of 50% mortality as gathered in blind studies. Supplement Figure 15B provides data comparing Replikin Counts of the four isolates with mean cumulative mortality as gathered in blind studies. The linear quantitative relationship between the predicted and experimental values is evident. U.S. Appln. Ser. No. 12/010,027 (published).

Table 27 below provides the histological data that was gathered for the moribund shrimp to demonstrate TSV infection. .

**Supplement Table 27 – Histology**
| UAZ ID#  | TSV Isolate | Days after exposure | TSV lesions¹ | LOS² |
|---------|-------------|---------------------|--------------|------|
| 06-407J/1 | Belize     | 3                   | G4           | G4   |
| 06-407F/1 | Thailand    | 3                   | G4           | G2   |
| 06-407D/1 | Thailand    | 4                   | G4           | G3   |
| 06-407E/1 | Thailand    | 4                   | G3           | G2   |
| 06-407A/1 | Hawaii      | 4                   | G2           | G3   |
| 06-407C/1 | Hawaii      | 4                   | G2           | G4   |
| 06-407H/1 | Venezuela   | 4                   | G4           | G2   |

Severity grade: G1: sign of infection; G2: moderate signs of infection; G3: moderate to high signs of infection; G4: severe infection.

1. TSV lesions = Presence of TSV pathognomonic lesions in the gills, mouth, stomach, intencuteral cuticular epithelium, and appendages.

2. LOS = presence of lymphoid organ spheroids within the lymphoid organ.

Belize TSV: Acute lesions of diagnostic TSV infection were found in one representative shrimp sample (06-407J/1) at a severity grade of G4. Nuclear pyknosis and karyorrhexis were observed in the cuticular epithelium of the general body surface, appendages, gills, stomach and esophagus. Lymphoid organ spheroids were also found at severity grade G4.

Thailand TSV: Severe (G4) TSV infection was detected in 2 out 3 shrimp (06-407D/1, F/1), another shrimp (06-407E/1) showed a moderate to high grade (G3) of infection. Lymphoid organ spheroids were found at severities of G2 and G3.

Hawaii TSV: Moderate level (G2) of TSV infection was detected in 2 shrimp (06-407A/1, C/1) collected at day 4. Lymphoid organ spheroids were found at severities of G3 and G4.

Venezuela TSV: Severe (G4) TSV infection was detected in one representative shrimp (06-407H/1) sampled at day 4. Lymphoid organ spheroids were found at severity of G2.

The real-time TSV RT-PCR assay was designed specifically for Hawaii TSV and thus a high level (10⁷ copies /µl RNA) of TSV was detected in the Hawaii-TSV challenged shrimp Table 28). The target sequence in 3 other isolates has 2 mis-matched nucleotides with the primers/TaqMan probe. Thus, there is 10 times less quantity of TSV (10⁶ copies/µl RNA) detected in Belize and Thailand samples. The Venezuela samples were detected with 100-100,000 times less: 10²-10⁵ copies/µl RNA; this may be due to both the effect of mismatches and a lower level of infection in the samples analyzed. Nevertheless, all 24 samples (6 from each isolates) were all positive for TSV infection. This confirms that the mortalities observed from bioassays are from TSV infection. The real-time TSV RT-PCR assay data is found below in the Table 28.

**Supplement Table 28 – PCR**

| TSV isolate  | Mean (Range) TSV copies/µl RNA |
|--------------|--------------------------------|
| Belize       | 2.7 x 10⁷ (4.8 x 10⁶ - 4.4 x 10⁷) |
| Thailand     | 2.7 x 10⁷ (4.3 x 10⁷ - 7.5 x 10⁷) |
| Hawaii       | 5.2 x 10⁷ (2.3 x 10⁷ - 7.5 x 10⁷) |
| Venezuela    | 6.5 x 10⁷ (6.5 x 10⁶ - 2.0 x 10⁷) |

**Laboratory Mortality Results Correlated With Replikin Counts**

Virulence of 4 TSV isolates (Hawaii, Belize, Thailand and Venezuela) was compared through a per os laboratory infection in juvenile *Litopenaeus vannamei* (Kona stock,
Oceanic Institute, Hawaii). The results showed that the Belize isolate is the most virulent, Thailand is the second, followed by the Hawaii isolate, and the Venezuela isolate is the least virulent. This is based on the analyses of cumulative survivals at the end of the bioassay (p<0.047) and the time when 50% mortality was occurred (p<0.001). That the mortality of the shrimp was caused by TSV infection was confirmed by positive reactions in RT-PCR detection and by the appearance of characteristic lesions observed in histological analysis.

**Laboratory Mortality Results Correlated With Replikin Counts**

Experimentally, Replikin Counts alone prospectively correctly predicted: (1) blind in controlled experiments in the laboratory, the order of lethality in shrimp of four strains of taura syndrome virus (Figures 15A and B); (2) an increasing H5N1 percent mortality in humans (Figure 4); and (3) the host (Figure 5); and (4) the country in which the latter would occur, Indonesia (Figure 6). For both H5N1 influenza in human hosts, and taura syndrome virus infection in shrimp hosts, evidence in this study demonstrates the quantitative relationship of the virus Replikin Count to the mortality rate in the host. The ability to predict blind is of course one of the more definitive proofs of a relationship; the demonstration of a quantitative linear relationship is even more definitive. Thus, the concentration of a class of specific virus peptides, Replikins, has here been quantitatively correlated with the percent mortality these viruses produce in their respective hosts, namely invertebrate crustacean (shrimp) and vertebrates (humans). To our knowledge, no quantitative correlation of virus structure and host lethality has been reported previously.

**REPLIKIN COUNT IN TSV EPIDEMIC**

An increase in Replikin concentration in taura syndrome virus (TSV) is predictive of an increase in virulence and lethality of the virus and allows for prediction of forthcoming outbreaks or increases in lethality.

Supplement Figure 19 illustrates a correlation between increased Replikin Count in the genome of TSV and outbreaks of the virus in 2000 and 2007 in shrimp. The Replikin Count data reflected in the graph is found in Supplement Table 19. Significant outbreaks of the disease are noted at years 2000 and 2007. It may be observed from the graph that outbreaks of the virus occur following an increase in Replikin concentration. In year 2000, TSV had a Replikin concentration of 2.7. Between 2001 and 2004, TSV had a lower mean Replikin concentration, as low as 0.7, and an identified Replikin Scaffold
disappeared. In 2005 the Replikin Scaffold reappeared, with an increase in lysines and histidines, and a commensurate increase in Replikin concentration to 1.8, followed by an increase in TSV outbreaks in 2006-2007.

**Supplement Table 19 - TSV Replikin Count**

| Year | PubMed Accession Number-Replikin Count | No. of Isolates per year | Mean Replikin Concentration per year | S.D. | Significance |
|------|----------------------------------------|--------------------------|-------------------------------------|------|--------------|
| 2000 | NP 149058 70 NP 149057 70 AAK72221 70 AAK72220 70 AAG44834 | 45 | 0.7 | 1.3 | low p<0.02 |
| 2001 | AAM73766 7 | 1 | 0.7 | 0.0 | prev p<0.02 |
| 2002 | AAN77089 2 AAN77088 2 AAN77087 2 AAN77086 2 AAW32934 | 28 | 0.7 | 0.4 | low p<0.50 |
| 2003 | AAR11292 6 AAR11291 6 AAR11290 6 | 3 | 0.6 | 0.0 | prev p<0.20 |
| 2004 | AAX07125 2 AAX07124 2 AAT81157 75 AAT81158 75 AAX07127 2 | 23 | 0.8 | 0.9 | low p<0.40, prev p<0.20 |
| 2005 | AAY56364 71 AAY56363 71 AAY44822 1 AAY44821 1 AAY44820 1 | 12 | 0.8 | 1.7 | low p<0.02, prev p<0.05 |

The TSV is less virulent than WSSV and the structure of the TSV Replikin Scaffold is less closely related to influenza virus than are the structures of WSSV Replikin Scaffolds.

**REPLIKIN CONCENTRATION IN REPLIKIN PEAK GENE OF RIBONUCLEOTIDE REDUCTASE GENE AREA CORRELATED WITH A WSSV EPIDEMIC**

An increase in Replikin concentration in white spot syndrome virus (WSSV) is predictive of an increase in virulence of the virus and allows for prediction of forthcoming outbreaks or increases in morbidity and, in extreme cases, mortality. A review of publicly available amino acid sequences of isolates of WSSV that demonstrate an increase in Replikin Count in the genome or a genome segment, or in a protein or protein fragment of the virus over time or between isolates is used as a predictor of an increase in outbreaks in shrimp. Publicly available sequences for isolates of WSSV from PubMed or other public or private sources may be analyzed by hand or using proprietary search tool software (ReplikinForecast™ available in the United States from REPLIKINS LLC, Boston, MA).

The authors have established a correlation between Replikin concentrations in WSSV and an increase in virulence of the virus resulting in epidemics. The authors reviewed publicly available amino acid sequences of isolates of WSSV having accession numbers at www.pubmed.com and have identified a remarkable increase in Replikin concentration in the Replikin Peak Gene of the ribonucleotide reductase gene area of the genome of the virus (e.g., SEQ ID NO: 669). The remarkable increase occurred just prior to a significant outbreak of WSSV in shrimp in 2001. Figure 18 illustrates a correlation between increases in Replikin Count in WSSV genome in 2000 and a significant outbreak of WSSV in 2001. In 2000, a remarkably high Replikin concentration of 97.6 is observed in WSSV. In the Replikin Peak Gene identified in ribonucleotide reductase in an isolate from 2000, the Replikin concentration spikes as high as 110.7, providing an unmistakable predictive signal for the significant 2001 outbreak of WSSV that followed. Analysis of the ribonucleotide reductase sequence.
A. Analysis of Annual Replikin Count of WSSV

The authors analyzed publicly available sequences for isolates of WSSV from PubMed. The data is contained in Supplement Table 18 and graphically described in Figure 18.

Mean Replikin concentrations were determined for all amino acid sequences for WSSV with accession numbers publicly available at www.pubmed.com. The mean Replikin Count was then determined for all viruses isolated and reported in a particular year. Supplement Table 18 provides the results of the Replikin Count analysis. Years with no data are not included in the table.

Prediction and Treatment of WSSV Outbreaks

Prediction of epidemics and future outbreaks may be made, for example, by reviewing the Replikin Counts of isolates of WSSV and comparing the Replikin Count for a particular year with Replikin Counts from other years. A significant increase in Replikin Count from one year to the next and preferably over one, two, three or five years or more provides predictive value of an emerging strain of WSSV that may begin an outbreak of more highly virulent WSSV. A WSSV outbreak may be predicted within about six months to about one year, to about three, to about five years or more from the observation of a significant increase in Replikin concentration. The outbreak is preferably predicted within about one to about three years and more preferably within about one to about two years. An outbreak of WSSV, therefore, may be predicted within 1 to about 2 years as demonstrated in Figure 18 wherein an epidemic occurred at about 1 year following a remarkably significant increase in Replikin concentration and in particular in the identified Replikin Peak Gene.

Significant increases may be observed over a time period of more than one year, such as three, four, five or more years. An outbreak may likewise be predicted within about six
months to about one year or more from the initial observation of an observable decrease in Replikin concentration following a notable increase.

The correlation between Replikin concentration and viral outbreaks noted above provide a method of predicting outbreaks of WSSV by monitoring increases or decreases in Replikin Count in the RPG of isolates of WSSV. The method may employ isolates of individual strains or isolates of all strains of WSSV.

In 2006 and 2007 WSSV has been observed to be dormant in shrimp. This continued decline of WSSV into “quiescent” or “dormant” levels in 2006-2007 is demonstrated in mean Replikin Counts for viruses isolated during 2005-2007 that are very low as compared to years wherein the virus demonstrated greater virulence, such as 2001. The continued quiescence in WSSV in 2007 may be contrasted with an observed rising of Replikin concentration in taura syndrome virus Replikin during this period. U.S. Appln. Ser. No. 12/010,027 (published).

Analysis of Replikin Counts in genomic and proteomic sequences alone prospectively correctly predicted: 1) the order of lethality in shrimp of four strains of taura syndrome virus (prediction was made blind in a laboratory study); 2) a 2007 increase in H5N1 percent mortality in humans; and 3) the country in which the increased percent mortality would occur most significantly, namely, Indonesia.

Counting of Replikin sequences within a malignancy, a virus, a protozoon, a plant or an animal is aided by computer review of databases of gene and protein sequences. Bacteria were accepted as real when the light microscope permitted them to be seen as discrete entities, sufficiently discrete that they could be counted. Similarly, viruses were accepted as real when the electron microscope permitted them to be seen as discrete entities, sufficiently discrete that they could be counted. Likewise, Replikins can now be accepted as real since the “computer microscope” permits them to be seen as discrete entities, sufficiently discrete that they can be counted. Hence, the Replikin Count, or determination of number of Replikin Sequences in 100 amino acids in any given genomic or proteomic sequence, is facilitated on a large scale by computer analysis and comparison of Replikin Counts has provided the necessary evidence to associate increased Replikin Counts (in both whole genomes and Replikin Peak Genes) with lethality.

Visualization and counting of Replikin sequences in a wide range of genomes has now revealed that Replikin sequences are not scattered throughout the genome of lethal, virulent and rapidly replicating entities but, instead, are concentrated in particular areas of the genome. The concentration of Replikin sequences in a particular area of the genome has now been identified as a Replikin Peak Gene (RPG). Concentration of Replikin sequences in a RPG provides a magnification of the Replikin Count and a magnification of the developmental, growth and disease associations with the presence of Replikin Sequences. The magnification effect of analyzing the Replikin Count of a Replikin Peak Gene as compared to Replikin Counts from other parts of a genome or the whole genome.
There, mortality in humans from H5N1 infection correlates strongly with an increase in Replikin Count in the pB1 gene area (RPG) of the virus while correlating less strongly with an increase in Replikin Count in the polymerase gene or the whole genome of the virus.

By means of visual and software inspection, 130,488 protein and genome sequences from common strains of influenza and other lethal viruses, isolated from 1917 to 2007 and accessible in PubMed were analyzed. Replikin sequences in these 130,488 sequences have been identified, counted and annually tracked. This extensive analysis revealed the Replikin Peak Gene that has now been found to be quantitatively related to lethality in several hosts, including plants, fish, crustacea and vertebrates, such as humans.

**Prediction of Pathogenic Outbreaks and Lethal Malignancies**

Prediction of epidemics and future outbreaks of viruses such as *Influenza A* (including H1N1, H2N2, H3N2, H3N8 and H5N1), foot and mouth disease virus, west nile virus, porcine reproductive and respiratory syndrome virus, porcine circovirus, white spot syndrome virus, taura syndrome virus, tobacco mosaic virus, coronavirus, and SARS virus, may be made, for example, by reviewing the Replikin concentration of isolates of a virus strain and comparing the Replikin concentration for a particular time period with Replikin concentrations from another time period. Prediction of outbreaks or increases in virulence or lethality of organism may also be made, for example, by reviewing the Replikin concentration of isolates of an organism and comparing the Replikin concentration for a particular time period with Replikin concentrations from another time period. Organisms for which outbreaks or increases in virulence and lethality may be predicted include, for example, *P. falciparum, M. mucogenicum* and *S. aureus*.

Significant increases may be observed over a time period of more than one year, such as three, four, five or more years. An outbreak may likewise be predicted within about six months to about one year or more from the initial observation of an observable decrease in Replikin concentration following a significant increase. U.S. Appln. Ser. No. 12/010,027 (published).

**Increased Replikin Counts in Replikin Peak Gene of pB1 Area of Influenza A Strains Correlates with Pandemics and Lethal Outbreaks**

The authors have identified Replikin Peak Genes as a segment of a genome, protein, segment of protein, or protein fragment in which an expressed gene or gene segment has the highest concentration of continuous, non-interrupted and overlapping Replikin sequences (number of Replikin sequences per 100 amino acids) as compared to other segments or named genes of a genome. The authors have likewise identified gene areas or proteins or protein fragments containing the highest concentration of continuous, non-interrupted and overlapping Replikin sequences (number of Replikin sequences per 100 amino acids) as Replikin Peak Genes.
Cycles in Influenza H5N1 and H9N2 Predict Expanding Virus Populations

The present discovery provides methods of predicting an increase in the virulence, morbidity, and/or lethality or an expansion of the population of an isolate of a strain of influenza virus as compared to another isolate or group of isolates of the same or a related strain. Such an increase may be predicted by identifying a cycle of Replikin concentration among a plurality of isolates of influenza and identifying a peak in that cycle. An increase is predicted following the time point or time period when the peak is identified.

A comparison of synchronized cycles of Replikin concentration in H5N1 and H9N2 may be seen in Supplement Figure 1. The synchronized cycles in these two influenza strains corresponds to and retrospectively predicts H5N1 outbreaks in 1997, 2001, 2004, 2007 and the present outbreak in 2008 and 2009.

![Figure 1](image_url)

Figure 1 illustrates two cycles of mean annual Replikin Counts in influenza sequences from the pB1 gene area for isolates isolated between 1993 and 2008 based on analysis of individual sequences reported at www.pubmed.com. The increase in mean annual Replikin Count corresponds to an increase in influenza outbreaks in flocks of poultry in Israel between 2000 and 2004. In Figure 1, the annual mean Replikin Count of the pB1 gene area of isolates of H9N2 is shown in black columns. The standard deviation data emphasize the extent of expanding Replikin Counts within the annual population. The number of poultry flocks reported in Israel with H9N2 infection is provided in white columns.

Figure 1 illustrates synchronous cycles of mean annual Replikin Counts in isolates of H9N2 influenza and isolates of H5N1 influenza in the pB1 gene area for sequences of
isolates isolated between 1993 and 2008 and reported at www.pubmed.com. Annual mean Replikin Count for H5N1 is reported in black columns with standard deviation reported above in white columns. Figure 1 visibly illustrates synchrony between the H9N2 and H5N1 Replikin Cycles. The synchronous cycles individually and together predict H5N1 outbreaks in Hong Kong in 1997, 2002, 2004, 2007, and a present outbreak of H5N1 and H9N2 in 2008-2009. Because the cycles of different strains correspond with a level of synchrony, the predictive capacity of the individual cycles is increased by the correspondence. Further an interrelationship between H5N1 and H9N2 is demonstrated suggesting that H9N2 may be a candidate for a future influenza pandemic just as H5N1 has been known to be a candidate for such a pandemic.

In Figure 1, the Annual standard deviation data emphasize the extent of the expanding Replikin Counts within the annual population and visibly illustrates synchrony between the H9N2 and H5N1 Replikin Cycles.

**Cycles in West Nile Virus Replikin Counts Predict Mortality and Geographical Expansion**

In a further aspect of the discovery, correlation between virus biochemical cycles and virus morbidity cycles are identified and used to predict increases in morbidity in a virus in a host population.

The following data in West Nile virus provides an example of cycling in mean Replikin Count in a virus wherein the cycle predicts morbidity. The data additionally further support immunogenic compounds, diagnostic compounds, and, among other things, vaccines because they support the principles upon which such Replikin vaccines and other therapies are based including, in particular, the role Replikin sequences play in virulence and morbidity in pathogenic diseases, the correlation of Replikin Count in diseases generally with pathogenicity, and the targeting of the Replikin structure in controlling rapid replication and disease.

Cycles are detectable because of repeating conserved virus structures and continuity of the Replikin phenomenon through time. The identified cycles provide a novel method of (1) determining the growth, spread, and path of an emerging disease, (2) predicting and tracking the occurrence and intensity of viral and other organism outbreaks by tracking changes in Replikin Count manually or using computer programs such as ReplikinsForecast™ (Replikins LLC) (3) designing and chemically synthesizing vaccines that contain both older conserved Replikins as well as newer ones to provide the most accurate and maximal anti-organism immune stimulating properties, (4) designing and chemically synthesizing antibodies that contain reactive sites against both older conserved Replikins and newer ones, to provide the most accurate and maximal anti-organism immune protective properties, and (5) designing and chemically synthesizing compounds that contain reactive sites against both older conserved Replikins and newer ones, to provide the most accurate and maximal anti-organism protective properties.
Supplement Figure 3 illustrates cycling of mean annual Replikin Count in West Nile virus in correlation with cycling of West Nile virus morbidity. Cycles are detectable because of repeating conserved virus structures and continuity of the Replikin phenomenon through time. The mean annual Replikin Count of the Envelope Protein of WNV (black) and standard deviation (capped line) is compared to the annual number of human cases in the United States as reported by the Centers for Disease Control (CDC) (gray). The standard deviation of the mean of the Replikin Count of the envelope protein is observed to increase markedly from 2000 to 2001 (p<0.001). This change has been observed in all common strains of influenza virus (not the same virus genus as WNV) to signal rapid replication and expansion of the range of the Replikin Count, thus virus population as defined by Replikin Count, preceding virus outbreak. The increase in the mean Replikin Count from 2000 to 2003 appears to accompany, or precede, the increase in the number of human WNV cases recorded independently and published by the CDC. A decrease in mean annual Replikin Count and recorded human cases of WNV is observed following 2003. In 2006, an increase is observed in the Replikin Count followed by an increase in 2007 of the number of human cases. As a result, two cycles of Replikin concentration and two cycles of WNV human morbidity are apparent, the first cycle from 2000 to 2003 and the second cycle from 2004 to 2006/2007.

Conclusion and Hypothesis:
The close relationship of genomic Replikin Count to morbidity and mortality throughout biology has led to the hypothesis that Replikins, in addition to being closely involved in the biochemistry of rapid replication, are in fact infective units, intimately related to both infectivity and lethality in viruses, bacteria, and plasmodia, and to lethality in cancer cells, and that these various hosts are merely carriers of the Replikin units.
Pandemic Prevention

Up to Two Year Prediction of Outbreaks and Decline of Malaria, Influenza, Infectious Salmon Anemia Virus, Foot and Mouth Disease Virus, and E. Coli also specify genomic Replikins sequences for solid phase synthetic vaccines, produced in 7 days, providing time and a realistic opportunity for pandemic prevention. <Replikins.com>

This communication is one of four submitted together:
Genome Replikin Count™ Predicts Increased Lethality of Resistant Tuberculosis
Genome Replikin Count™ Predicts Increased Lethality of Malaria
Genome Replikin Count™ Predicts Increased Lethality of Cancer
Genome Replikin Count™ Predicts Increased Infectivity/Lethality of Viruses