Genomic Replikin Counts™ of Infectious Salmon Anemia Virus (ISAV) in Canada Exceed the Counts in Lethal Outbreaks in Norway, Chile, and Scotland.

Real-Time Tracking of the Evolution of the ISAV Genome and the Resultant Replikins Solid Phase ISAV Vaccine Make ISAV Pandemic Prevention Possible.

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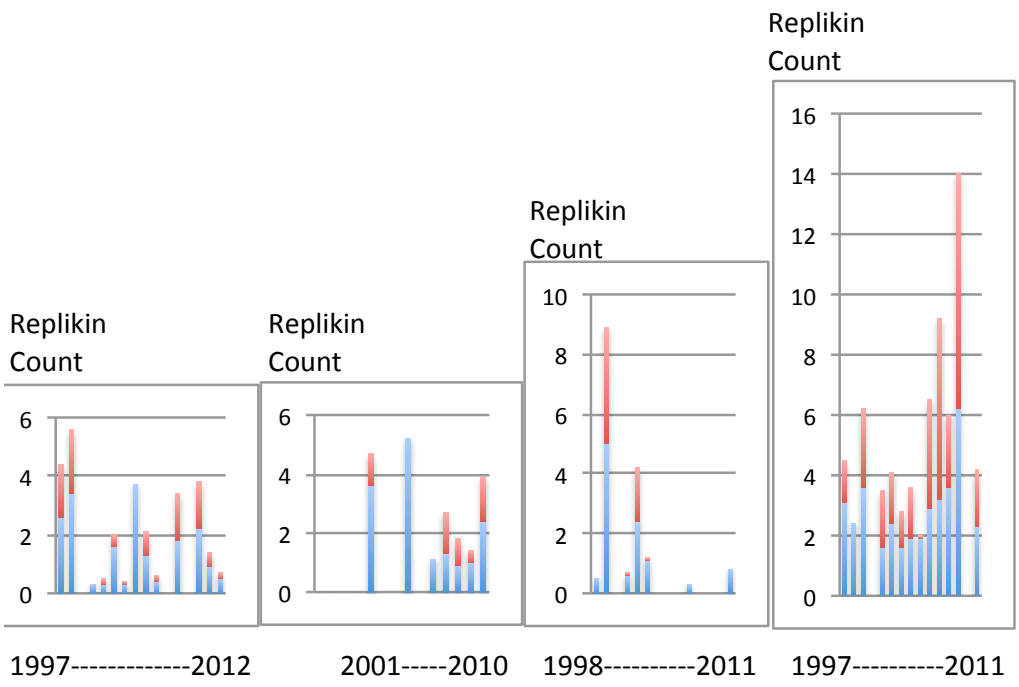
FIGURE - ANNUAL ISAV REPLIKIN COUNTS IN FOUR COUNTRIES

Mean,blue; Standard Deviation,red



LEGEND: All ISAV sequences listed on Pubmed were analyzed for replikin Counts and are included in this Figure.

The highest Replikin Count in Norway, in 1998, was followed by a major outbreak of ISAV in 1999; in Chile,the highest Count, in 2004, was followed by the outbreaks in 2005, and high Counts in 2007 and 2010 were followed by outbreaks in 2009 and 2011 (18); in Scotland, the highest Count in 2009 accompanied the outbreaks in 2009; In Canada, the highest ISAV Replikin Count occurred in 1999, which is likely relevant to the marked loss of salmon in Canadian waters in 2010 (10,19). The size of the Standard Deviation of the Mean indicates the number of viruses in a given population with Replikin Counts higher than the Mean, and thus the proportion of the viruses that year engaged in rapid replication (1). In Influenza A, a member of the orthomyxovirus family as is ISAV, rapid replication invariably accompanies, or precedes outbreaks by as much as two years (1-8).

Summary

Advance warning of pathogen outbreaks, such as those by influenza and ISAV, both members of the orthomyxovirus family, has not been possible heretofore. A new class of genomic peptides associated with rapid replication was discovered and named Replikins. Software was designed to analyze Replikins quantitatively. Replikin concentration changes were measured annually prior to, and "real time" every few days during, the 2009 H1N1 influenza pandemic. Replikins were seen by both linear sequence representation and three-dimensional X-ray diffraction, and found to increase in concentration from Replikin Counts of 3.2 to 5.5 to 10.1 and to expand on the virus hemagglutinin surface prior to and during the H1N1 pandemic. A highly significant increased concentration of virus replikins was found to correlate with influenza virus outbreaks a) retrospectively in three pandemics from 1918 to 1999 (14,227 sequences)($p<0.001$), and b) prospectively before the H1N1 2009 pandemic in the hemagglutinin gene, p values by t-test = $1/10^{130}$, 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 = $2/10^{22}$. Rising replikin concentration in H1N1 from 2006 to 2008, predicted one year in advance the H1N1 outbreak of 2009; and in H5N1, predicted the lethal outbreaks of H5N1 1997-2010 (1-7). We then found (8) that the area in the genome of the highest concentration of Replikins, and the country in which this peak exists in scout viruses, permitted in the past five years seven consecutive accurate predictions of the geographic localization of coming influenza outbreaks, including those now realized in Mexico for H1N1, and in Cambodia for H5N1. Real-time Replikin analysis of the evolution of the virus genome identified both mutations and structural reorganization of the hemagglutinin and p B1 genes over as much as two years before each outbreak. This information, together with the specific Replikin sequences so obtained, permitted solid-phase synthesis of Replikin vaccines in seven days, which blocked H5N1 in chickens (11). The information also now provides up to two years of time to thoroughly test and distribute vaccines to high risk animals and humans in the countries identified; thus for the first time, a quantitative genomic Replikins method is available to both predict initial outbreaks and to prevent the development of a pandemic. This new technology is now also being applied to outbreaks of another member of the orthomyxovirus family, Infectious Salmon Anemia Virus (ISAV).

Methods

Software based on the authors' algorithm (1) first identified and then counted the replikin peptides in each genomic sequence (Replikin Count™ = number of replikins per 100 amino acids). For each annual group of sequences, the Replikin Count™ mean and standard deviation of the mean (SD) were calculated and compared with other years. Highly statistically significant increases and decreases were examined, for example by

strain, host, country, history, year, month or week; by substitution, morbidity, and lethality. The terms 'increase' and 'decrease' of Replikin Counts™ were used only when the p level was less than 0.001. All Counts for all sequences on Pubmed were each monitored separately, from 1997 to 2012, for all countries reporting to Pubmed. Statistical analyses of rate of change, trend, pattern, and growth models in the evolution of each virus strain were initiated. Replikin genes were isolated *in silico* by scanning and identifying those areas of the virus genome which had the highest concentration of replikins. The Count was compared with the Count in other countries in the same year, and with the occurrence of outbreaks (Figure). Replikin peptides were visualized by two means: a) by linear display of sequences of contiguous numbered amino acids in the primary structure and b) by X-ray diffraction analysis of the 3-dimensional folded structure, which showed the increased coverage by Replikins of the HA gene surface in H1N1 real-time as the Replikin Count™ increased from 3.2 to 5.5 to 10.1 from before and during the course of the H1N1 pandemic of 2009 (1).

Results and Discussion

The Replikin Counts® of Infectious Salmon Anemia Virus (ISAV) lethal Replikin genes have increased since 2005 ($p<0.001$) to mean Counts greater in Canadian salmon than those found during highly lethal ISAV outbreaks in Norway, Chile, and Scotland (Figure). The Replikin Count™ equals the number of Replikins per 100 genomic amino acids specified. In addition to the significance of the mean annual Count and the standard deviation of the mean, as discussed in the Legend to the Figure above, the peak individual specimen Replikin Counts reached (not shown) were also instructive as markers of the progress of the evolution of the virus. The peak individual Counts observed to date in all Pubmed ISAV data are 5.6 in Chile, 7 in Norway, 9.4 in Scotland, and 22.6 in Canada. Replikin Count™ increases correlate with increased lethality in several classes of infectious disease, in influenza viruses, in Taura Syndrome virus in shrimp, in other viruses, in bacteria, and in plasmodia (1-8). The Replikin Count has been used to predict the relative lethality of shrimp Taura Syndrome Virus (TSV) isolates, the accuracy of the prediction confirmed in the linear relationship observed in the laboratory. Replikins TSV Synthetic Vaccines have been shown in the laboratory to block TSV infection in shrimp (12). Regional temporal cyclic expansion preceded by increased concentration of Replikins observed in other viruses (9) also may be occurring for ISAV, as seen in the Figure.

The present data suggest that the marked decline in salmon yields in recent years in Canadian and U.S. waters (10,19), at least in part, may be related to ISAV. ISAV is acknowledged to exist on Canada's Atlantic coast; but there has been some disagreement expressed about the presence of ISAV in Pacific Canadian salmon farms in British Columbia, whether it has reached salmon in the Fraser River, and whether it is a lethal strain. Some government ISAV data apparently has not yet been completely published (14,19). The situation in British Columbia could also impact the Alaska salmon fisheries, should the virus be found spreading among wild stocks.

To counteract this risk of spreading, a solid phase synthetic ReplikinsISAV™Vaccine has been formulated, and is now available for trials, based on

ISAV Replikins genomic structures conserved back to 1997. The ISAV vaccine is similar in its basis to the oral Replikins TSV Vaccine, and to the TransFlu™Vaccine which has been demonstrated to block H5N1 virus and its excretion by chickens (12). For the first time it may be possible to counter virus reservoir formation where mutation can occur to more lethal strains (12). The ReplikinsISAV™Vaccine will be applied in the same way to counter reservoir formation in Canada and elsewhere.

Lack of basic information and transparency hinder progress in solving the challenge of ISAV, as noted both in Canada (10,14,19) and earlier in Chile (18). The absence of published data at particular times, some critical for interpretation, is evident in the Figure.

Salmon and other fish farms are an increasingly important source of food in many areas of the world. Outbreaks of Infectious Salmon Anemia Virus (ISAV) are responsible for large losses in salmon farms. Lethal ISAV in Chile is reported to have resulted in over $2 billion dollars in losses in 2010. ISAV belongs to the family *Orthomyxoviridae*, together with influenza viruses but is sufficiently different to be assigned to its own genus, *Isavirus*. Some ISAV strains appear to be relatively 'benign' (17), sometimes referred to as salmon 'flu', and apparently produce no lethal disease. But no quantitative methods have been available previously to distinguish genomic sequence structures which are benign from those which relate to lethality, nor which indicate when apparently benign strains mutate to lethal ones. Consequently, salmon farmers in Chile who have salmon pens infected with any ISA strain other than HPR0 (although its allegedly benign disposition may be changed by rapid replication and mutation), are required to kill their fish, and they have no way to tell in advance if the strain they are dealing with is lethal or not. This also makes the exclusion of lethal strains of ISAV in salmon eggs impossible when deciding which to use to stock new salmon aqua farm pens. The determination of Replikin Counts™ now should help in defining the degree of lethality, as they have been shown to be able to do with Taura Syndrome Virus in shrimp (11).

The high Replikin Counts™ of ISAV in Canada serve as evidence supporting ISAV as a cause of salmon loss currently, as well as a warning of the risk of outbreaks to come. The risk may be substantial in view of the accuracy of the Replikin Count predictions in seven successive instances of lethal outbreaks over the past five years by ISAV's related orthomyxovirus, influenza, in all of which not only the prediction of the strain-outbreak (as discussed by the UN Food and Agriculture Association (3)), but also the geographic location of the outbreaks (8), were correctly predicted. Replikin studies on ISAV are discussed in greater detail in a book in preparation, *The Evolution of Lethal Replikins* (7).

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