# Model on Potential Enhanced Virulence of Influenza A H1N1: A Possible Implication of 2',3 Diphosphoglycerate Pathways in Immediate Early Events Leading to Host/ Viral Membrane Fusion. Prospective Clinical Benefit of Erythropoietin.

Dr. Gabriel Pulido-Cejudo* & Dr. Abraham Pulido-Cejudo
International Centre for the Advancement of Health Regional Innovation & Science
Dr. Balmis 148 Col. Doctores 06720 Pabellón 307
28-Apr-09 Ottawa, Canada
*Corresponding Author

**Although the actual geographical location of the first case of North American Influenza remains unsettled, the recent swine flu outbreak in Mexico apparently originated in the State of Veracruz within the South East Coast of the country.**

The apparent most predominant subtype of Influenza A during this recent outbreak has been identified as H1N1 [WHO, April 2009]. Nonetheless, this is not exclusive of other reassortant viruses, hence justifying continuous surveillance.

It is possible that some other reassortant viruses such as those that have appeared through reassortment between traditional H1N1 and H3N2 subtypes and isolated primarily from pig farms in Korea might also emerge [Shin, Song, Lee, Lee, Kim, Kim, Choi, Kim, Webby and Choi, 2006].

These could include the fairly new reassortant H3N1 virus isolated from coughing pigs (A/Swine/Minnesota/00395/2004) identified as a result of phylogenetic analyses of Hemagglutinin segments (comparable to cluster III H3N2 SIVs) and neuraminidase sequence of the currently prevalent H1N1 [Ma, Gramer, Rossow, and Jin Yoon, 2006].

In view of interspecies transmission and the importance of animal infections acting both as viral reservoirs (waterfowl and seabirds) or in the evolution of human influenza A viruses (pigs) [Karasin, West, Carman and Olsen, 2004], some countries have taken extreme and somewhat debatable measures to prevent a fast pace evolution and emergence of more virulent strains of Influenza A (Figure 1). These measures have been justified as a sound and effective approach to prevent future pandemic viruses.

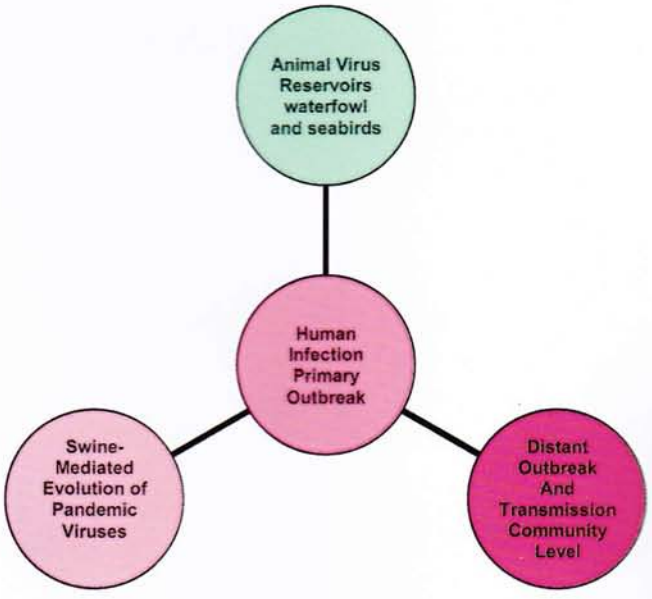


**Figure 1. Reservoirs, Viral Reassortment, Interspecies Transmission and Seasonal Disease Outbreaks.** Human Influenza A virus subtypes are phylogentically related to those found in bird reservoirs. However, reactive epitopes from reference swine influenza viruses are distinctively different from human viruses. Interspecies transmission is essential to the evolution of human influenza viruses [Karasin, West, Carman and Olsen, 2004].

Based on the pattern of transmission and rapid spreading of the recent outbreak that commenced in Mexico during late March early April 2009, the World Health Organization (WHO) raised worldwide alertness to Level 5 (human-to-human spread of the infectious pathogen in at least two countries of a WHO region).

It equally warned that if the trend were to be continued, the current Level 5 could mount into a potential Level 6 (commencement of a pandemic with community spreading of the disease through human-to-human viral transmission in at least 3 countries of a WHO region; see Figure 2) [WHO, April 2009]. This implies that apart from continuous and intense surveillance, response and mitigation efforts are sought.

In this short manuscript a model to architecture a potential clinical response aimed at mitigating the mortality caused by the current H1N1 outbreak is provided.

## Current Status and Prime Causative Infectious Agent Detected

The current onset of swine flu has been characterized by acute respiratory distress causing death. Influenza A subtype H1N1, has been identified as the prime causative agent of the current outbreak. During the course of the last two weeks, the infection has spread to Canada, The United States the United Kingdom and Spain among other countries around the world. As of to date, 16 countries have reported confirmed cases of H1N1 Influenza A outbreaks.

Fatalities have also been reported outside of Mexico primarily in Texas within the United States. Taking into account the current level of infectivity rates and disease spreading, earlier this week the World Health Organization alerted that a potential pandemic might be imminent. However, based on the relative attenuation of infection rates and virulence of H1N1 detected in Mexico during the past weeks, a pandemic seems less likely.

According to various communications provided by the Mexican Embassy of Mexico in Canada [S. Jacobo, personal communications, April 30, 2009] and a recent press conference by the Secretary of Health in Mexico (April 27, 2009), close to 160 deaths have been confirmed as result of H1N1 infection. In addition, out of 2000 patients that were hospitalized, the majority of patients are from Mexico City, the State of Mexico and the state of San Luis Potosí. According to the same report approximately 53% of previously hospitalized patients have been released, a trend that continues to improve.

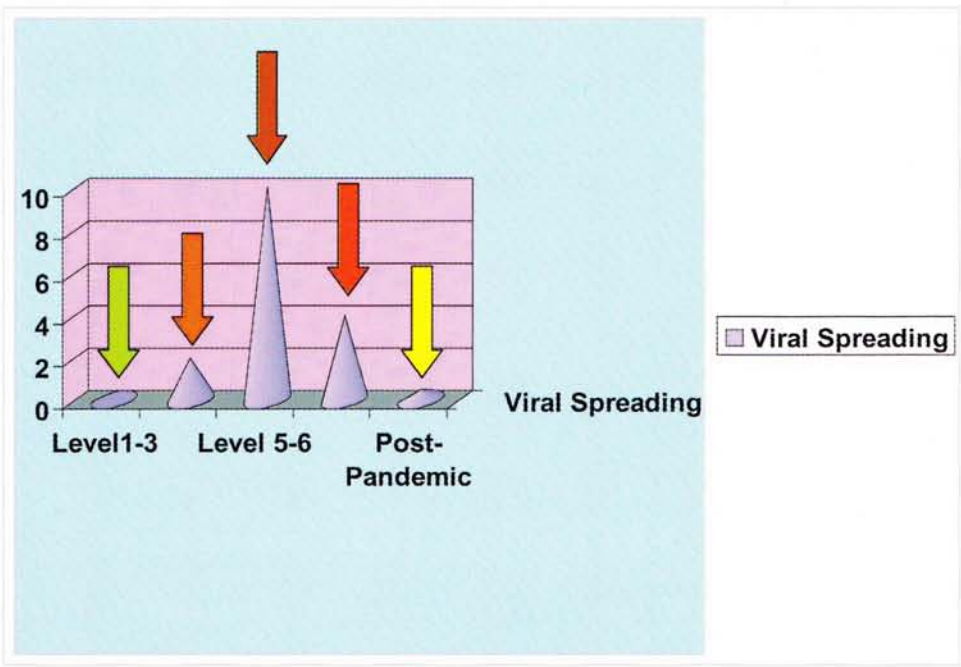


**Figure 2. Schematic Representation of WHO Influenza Alerting System**

- Occurrence of namely animal infections. Few human cases (1-3).
- Regular human to human infections (4).
- Uncontained human infections in various regions far from initial site of infection (5-6).
- Infections occurring post-maximum levels attained.
- Post-pandemic. Regular levels of seasonal rates of infection.

Nonetheless, the current pattern of differential infection leading to enhanced mortality within Mexico, particularly within Mexico City, the State of Mexico and San Luis Potosí by comparison to other cities and countries affected by H1N1 outbreak, has sparked a stern interest in determining the contributing factors that could explain this asymmetrical occurrence of infection and most importantly in mortality.

In view of the relatively early reporting and lack of thorough clinical analysis and evolution of the outbreak, medical officials, scientists and epidemiologists have provided different potential explanations for this apparent asymmetry in incidence and mortality.

As of to date some have simply dismissed any significant relevance of this phenomenon assuming that the higher concentration of cases in Mexico City, the State of Mexico

and San Luis Potosí, as well as the relatively less acute symptoms experienced in patients affected outside of Mexico is irrelevant and perhaps simply reflecting the rapid mutation of the current H1N1 strain into less severe forms as it reaches other States in Mexico and other countries.

Other equally informal and temporary explanations provided relate to the potential immune-compromised profile of malnourished populations in developing countries which has been identified as one of the reasons of the incidence of measles in children younger than 5 years of age by comparison to the incidence observed in developed countries which takes place in children between 5 to 10 years of age [Brooks, Butel and Morse 2007]. Other considerations vary from lack of adequate medical attention, limited access to effective medical treatment and follow up to pollution levels and ineffective surveillance.

While it is difficult to objectively single out one major aspect, to effectively address the asymmetry in H1N1 virulence during the current Level 5 outbreak affecting primarily the Mexican Population in three major States, it provides a unique opportunity to address yet undocumented possible metabolic pathways that could contribute to a particular higher susceptibility towards Influenza A H1N1 subtype and perhaps other closely related strains.

## Asymmetrical Incidence of Influenza A H1N1 and Number of Fatalities

Regardless of the current level of disease spreading involving the same Influenza A H1N1 subtypes, it appears that the number of fatalities detected in Mexico City, The State of Mexico and San Luis Potosí are greater than in any other region affected by this disease outbreak. Amongst the various possible factors contributing to an apparent increase number of deaths, it is possible that a greater level of virulence is attained in view of the relative higher altitude and population density in these States, particularly in Mexico City by comparison to other countries affected.

Briefly, Mexico City has a relative altitude of 2,235 metres above the sea; Toluca within the State of Mexico locates at 2,680 metres and San Luis Potosí at 1300 metres from sea level. Although it remains too early to confirm any significant asymmetry in the incidence of Influenza A H1N1 in the current outbreak affecting Mexico and 16 countries in various geographical regions, according to official sources [S. Jacobo, personal communications, April 30, 2009; Secretary of Health of Mexico, May 2, 2009] Mexico City, The State of Mexico and San Luis Potosí are the most affected areas.

Apart from their higher altitude, these regions are most likely the most densely populated in Mexico and in some cases, amongst the world.

Another asymmetrical difference concerning H1N1 outbreak appears to be gender related whereby at least in Mexico, more women than men seem to have been affected by the disease.

## Model & Potential Clinical Implications: Altitude and Gender Related Asymmetries of 2',3 Diphosphoglycerate Levels and Metabolism

It has been fairly well documented that 2',3 Diphosphoglycerate levels augment even at moderate higher altitude levels by comparison to those detected at sea level [Klausen, Ghisler, Mohr and Fogh-Andersen 2007]. Such an increase is concomitant with a higher production of Erythropoietin. Both processes lead to a swift compensation of oxygenation rates. 2',3 Diphosphoglycerate induces a higher rate of release of oxygen transported by haemoglobin while Erythropoietin enhances the production of red blood cells in response to hypoxia.

In addition to altitude induced-hypoxia, it appears that gender related differences lead to physiological adaptations to anaemia amongst female population including efficient shifting of haemoglobin oxygen dissociation rates by reduced haemoglobin oxygen affinity in response to higher levels of 2',3 Diphosphoglycerate [Duncan and Levin, 2005].

## Potential Role of 2',3 Diphosphoglycerate in Enhancing Influenza A Entry and Infectivity

Although it has been extensively postulated that the mechanism of Influenza virus entry into host cells takes place through clathrin-polimerization mediated endocytosis amongst other possible endocytic pathways [Lakadamyali, Rust and Zhuang, 2004], the exact mechanism leading to viral entry remains a challenging undertaking.

Recent studies concerning the absence of clathrin endoctosis implies that other alternative pathways might coexist leading to the speculation that other intermediate processes might be crucial during the immediate early events of binding and viral entry [Rust, Lakadamyali, Zhang and Zhuang, 2004].

It is possible that apart from specific receptor-binding, other metabolites and cell surface molecules might facilitate Influenza viral fusion in a similar fashion as that previously reported in the case of unrelated viruses such as that of HIV viral entry [Pulido, Conway, Proulx, Brown and Izaguirre, 1997].

Based on the possible implication of accessory molecules capable of enhancing Influenza A targeting towards cellular endocytic apparatus, a potential synergistic role of 2',3 Diphosphoglycerate in the immediate early events of Influenza A virus entry could be postulated.

The possible implication of 2',3 Diphosphoglycerate could potentially explain in part the asymmetrical H1N1 virulence during the current Level 5 outbreak affecting primarily the Mexican Population in three major states (Mexico City, The State of Mexico and San Luis Potosí) in view of their considerable higher altitude above sea level in addition to environmental factors such as undetermined exacerbating air pollutants. In addition, due to the differential gender responses induced by 2',3 Diphosphoglycerate this could also address in part the apparent higher incidence of Influenza A in Mexican women.

## Presumed Role of 2',3 Diphosphoglycerate in Immediate Early Events During Viral Targeting Towards Host Endocyitc Apparatus

Viral entry is a multi-stage process that in immediate early events following receptor-mediated binding to the surface of healthy host cells, co-receptor binding events lead to a cascade of trans-membrane signalling mediated progressive morphological changes [Lakadamyali, Rust and Zhuang, 2004; Chou and D'Orsogna, 2007].

During these early events, cytoskeletal proteins have been claimed to play a pivotal role towards nascent fusion pores driven by a well-characterized fusogenic agent, the influenza virus hemagglutinin [Richard, Leikina and Chernomordik, 2008].

Although, the exact role of the cytoskeleton and its re-organization in pore expansion remains unclear, influenza hemagglutinin mediated local fusion to syncytium requires metabolic activity of living cells [Richard, Leikina and Chernomordik, 2008].

According to early membrane fusion models, it has been found that 2',3 Diphosphoglycerate, might modulate the size of the fusion zones as well as that of their time-dependent expansion [Wu, Rosenberg and Sowers, 1994].

Other factors such as wheat germ agglutinin were also shown to intensify early events of fusion zone diameter [Wu, Rosenberg and Sowers, 1994] suggesting that the immediate early dynamic events leading to cell membrane fusion might depend upon potential micro-environmental changes, particularly sensitive to that of 2',3 Diphosphoglycerate levels.

The micro-environmental changes on the cell membrane induced by 2',3 Diphosphoglycerate appear to be mediated through its specific binding to cytoskeltal proteins such as Spectrin [Sheetz and Casaly, 1980; Wu, Rosenberg and Sowers, 1994], Actin and Tubulin [Lebbar, Stetzkowski-Marden, Mauffret and Cassoly, 2004].

The potential modulation of cytoskeletal proteins through 2',3 Diphosphoglycerate binding could lead to exploring its role in both non-Clatherin and Clatherin-mediated viral entry of Influenza A H1N1 subtype. As such, positive modulation of immediate early viral events leading to receptor-independent enhanced viral adsorption and membrane-host fusion could be triggered by higher micro-environmental concentrations of 2',3 Diphosphoglycerate.

Even though 2',3 Diphosphoglycerate is predominantly found within erythroid and enucleated red blood cells at a ratio of approximately 1000 fold more than that measured in non-erythroid cells, most cells contain quantifiable amounts of this important metabolite [Yeoh, 1980].

As in the case of erythrocytes and erythroid precursors, hypoxia also enhances 2',3 Diphosphoglycerate in non-erythroid cells most likely through its direct effect on glycerldehyde-3-phosphate dehydrogenase [Yamaji, Fujita, Takahashi, Yoneda, Nagao, Masuda, Naito, Tsuruo, Miyatake, Inui and Nakano, 2003]

Sialyloligosaccharides within the human respiratory epithelium are specifically recognized by Influenza virus strains, whereby an increase in 2',3 Diphosphoglycerate in response to hypoxia could translate into a higher membrane-host fusion of selectively adsorbed viral particles [Nelson, Couceiro, Paulson and Baum,1993].

## Prospective Role of 2',3 Diphosphoglycerate Levels in Influenza A Asymmetric Infectivity and Mortality Caused by H1N1 Recent Outbreak

Apart from the rapid and unexpected emergence and virulence of Influenza A H1N1 primarily affecting Mexico, the apparent gender and geographic asymmetry, left medical officials and health professionals speculating on the possible propelling factors that could explain the higher incidence and mortality in Mexico City, the State of Mexico and San Luis Potosí, as well as the perceptible higher mortality amongst women.

Although the aforesaid regions share a higher density population, they also locate at higher altitudes by comparison to other urban cities within Mexico (Mexico City 2,235 metres above the sea; Toluca within the State of Mexico locates at 2,680 metres and San Luis Potosí at 1300 metres respectively).

Whether the early detected gender and geographic asymmetry of the recent H1N1 outbreak in Mexico is corroborated or not, the current observations support the notion that hypoxia-induced higher 2',3 Diphosphoglycerate levels might be singled-out as a viable explanation to the differential virulence and infectivity of H1N1.

Higher altitude and air pollutants can trigger elevated concentrations of 2',3 Diphosphoglycerate levels with different metabolic patterns amongst women and men. Based on the positive modulation of 2',3

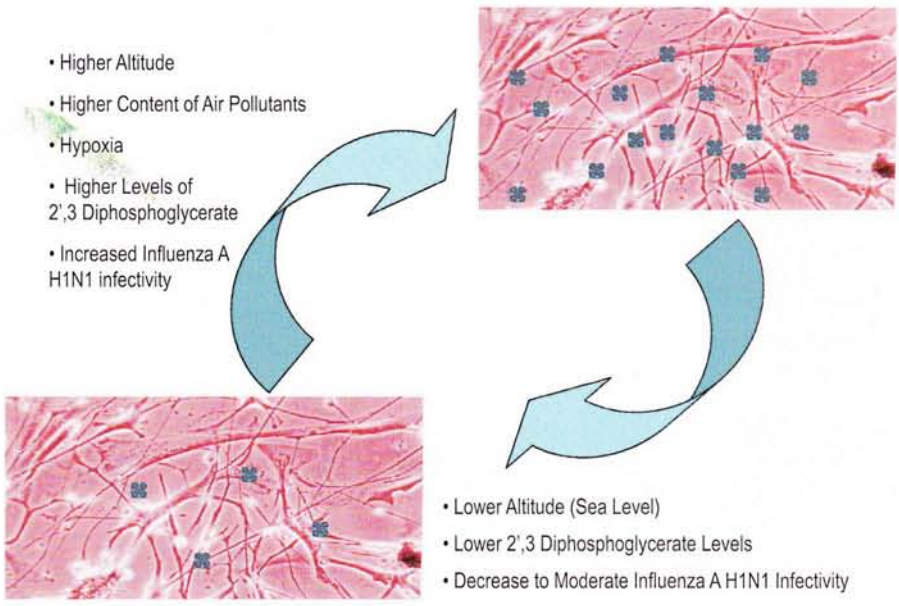


**Figure 3. Higher altitude and air pollutants can trigger elevated concentrations of 2',3 Diphosphoglycerate levels with different metabolic patterns amongst women and men.** Based on the positive modulation of 2',3 Diphosphoglycerate on cytoskeletal proteins and immediate early events of viral adsorption and enhanced membrane-host fusion, it is possible to speculate on the possible role of micro-environmental 2',3 Diphosphoglycerate on a higher virulence and infectivity rates of Influenza A H1N1. As depicted on Figure 3, higher levels of 2',3 Diphosphoglycerate in response to hypoxia could translate into a higher infectivity rates.

Diphosphoglycerate on cytoskeletal proteins and immediate early events of viral adsorption and enhanced membrane-host fusion, it is possible to speculate on the possible role of micro-environmental 2',3 Diphosphoglycerate on a higher virulence and infectivity rates of Influenza A H1N1 (Figure 3).

Formal experimental and clinical data in support of this working model can not solely explain the apparent gender and geographical asymmetry of the recent Influenza A H1N1 outbreak in Mexico. The potential implication of 2',3 Diphosphoglycerate on an enhanced Influenza A H1N1 infectivity rate, could also lead to the possible inclusion of an early clinical intervention based on Erythropoietin treatment.

Erythropoietin administration as an adjuvant therapeutic approach to rescue non-respondent patients could reduce hypoxia as well as micro-environmental concentrations of 2',3 Diphosphoglycerate. This in turn could lead to a significant decrease on viral infectivity, a better clinical management of infected individuals and overall patient outcome.

Possible involvement of 2',3 Diphosphoglycerate in the immediate early events of Influenza A viral entry could signal the primordial mechanistic events that through sustained animal infections and interspecies transmission must have permutated into a more complex processing and diversity of modern mechanistic events postulated through non-clatherin mediated infection as well as clatherin-mediated viral endocytosis.

The reported effect of 2',3 Diphopsphoglycerate on cytoskeltal peptides signifies that although various anchoring cell-surface receptors could be involved at any given time of the constant evolution of Human Influenza A viruses, the 2',3 Diphosphoglycerate-dependent routing would have remained a universal master key and viral port of entry.

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## Post Publication Update

In an unprecedented scientific collaborative effort, between Canada and Mexico, on May 7, 2009, scientists under the direction of Dr. Frank Plummer, Scientific Director General of the National Microbiology Laboratory in Winnipeg , sequenced the entire H1N1 genome from virus samples from both countries.

During a press conference on May 7, 2009, and having deposited the genetic sequences of three viruses within the Public Database GenBank (http://www.ncbi.nlm.nih.gov/genomes/FLU/SwineFlu.html), the collaborative Team of Scientists claimed that the sequences appeared to be a genetic match with no specific genomic sequences that could explain the increased virulence of H1N1 detected during the outbreak in Mexico.

This enhances the paramount importance of an intense search of host and environmental factors such as the potential role of 2',3 Diphosphoglycerate on cytoskeletal peptides and the immediate early events leading to increase viral entry of Influenza A H1N1.

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