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# Age wise Comparative Positivity Rate of H1n1 Using Rt- Pcr in The Year 2011- 2012

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***ABSTRACT:*** *The emergence and rapid spread of the 2009 H1N1 pandemic Influenza A virus (H1N1 pandemic) in humans highlights the importance of enhancing the capability of existing Influenza surveillance system with tools for rapid identification of emerging and re-emerging viruses. One of the new approaches is the RT-PCR Technology; which is based on analysis of base composition (BC) of RT-PCR amplicons. Samples were collected from different collection centers and the specimen type taken was throat and nasal swab in VTM. About 324 samples are collected from different collection centers in Delhi NCR and Jaipur. Further isolating of RNA with Qiagen kit and detecting the specific targets for H1N1 virus. All samples was examined by RT-PCR Technology in which 2.46% samples showed H1N1 positive result among which 2.84% were Males and 2.03% were Females showing that male are more susceptible to this infection rather than Females, while the remaining are negative for H1N1 and out of the remaining sample examined 9.57% were found to be infected by Influenza A virus only among which 9.65% were Males and 9.45% were Females showing that in case of Influenza A virus also Males are more susceptible than Females. It means that, in both cases males are more prone to Influenza virus infection.*
*The RT-PCR assay is a broad range Influenza virus identification tool that can be used directly on clinical specimen for rapid and accurate detection of Influenza virus genes. The assay differentiates the H1N1 pandemic from seasonal and other non human host virus.*

## I. INTRODUCTION

Swine Influenza (swine flu), first isolated from a pig in 1930, is a respiratory disease of pigs. It is caused by type A Influenza Virus (H1N1 subtype), which is the only type of Influenza virus to have caused pandemics. Swine flu outbreaks in pigs occur regularly, causing high levels of illness and low death rates. Swine Influenza viruses may circulate among swine throughout the year, but most outbreaks occurs during the late fall and winter months similar to outbreaks in humans. Swine flu occasionally infects people without causing large outbreaks. Only twelve cases of swine flu were reported in the United States over the last four years (January 2005 through January 2009). None of them caused deaths.

An outbreak of swine flu occurred among soldiers in Fort Dix, New Jersey, in 1976. At least four soldiers became ill with swine flu and one died; all of these patients had previously been healthy. The virus was transmitted to close contacts in a basic training environment, with limited transmission outside the basic training group. The virus is thought to have circulated for a month and disappeared.The Influenza virus belongs to Orthomyxoviridae family. It has three classes: A and B which only infect humans and C which is uncommon. Its genetic material is made up of eight separate segments. The virus is enveloped with two important projections on the surface; these are haemagglutinin that binds to cell receptors in target tissues and Neuraminidase that cleaves to the sialic acid in the cell wall to release the progeny viruses. Influenza A has 16 different haemagglutinins and 9 different Neuraminidases. It is classified according to the types of haemagglutinin and Neuraminidase on its surface, e.g. H1N1, H3N2 and H5N1.

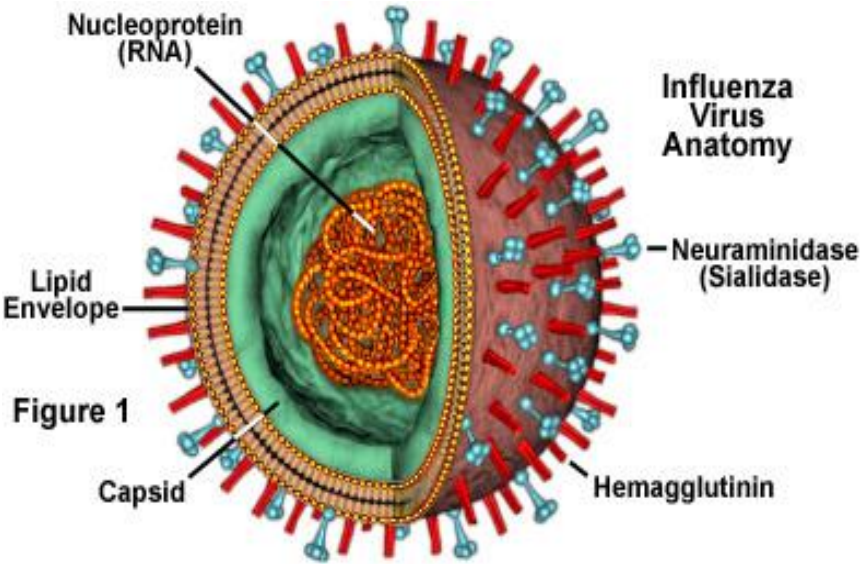



**Fig 1:** Structure of InfluenzaA virus

Like all Influenza viruses, swine flu viruses change constantly. Pigs can be infected by avian Influenza and human Influenza viruses as well as swine Influenza viruses. When Influenza viruses from different species infect pigs, the viruses can reassort (i.e. swap genes) and new viruses that are a mix of swine, human and/or avian Influenza viruses can emerge. Over the years, different variations of swine flu viruses have emerged. At this time, there are four main Influenza type A virus subtypes that have been isolated in pigs: H1N1, H1N2, H3N2, and H3N1. However, most of the recently isolated Influenza viruses from pigs have been H1N1 viruses. The epidemic situation of A H1N1 flu arose in North America in April 2009, which rapidly expanded to three continents of Europe, Asia and Africa, with the risk ranking up to level five. Until May 13th, the flu virus of A H1N1 had spread into 33 countries and areas, with a laboratory confirmed case number of 5728, including 61deaths.(Zheng *et al*; 2009)

On 17th April 2009, the Center of Disease Control and Prevention (CDC), in the USA, reported Influenza A H1N1 strain with quadruple segment translocation in its RNA. On11th June 2009 it was declared by the World Health Organization (WHO) to be a Phase 6 pandemic virus (maximum threat).This was the first declared flu pandemic in 41 years. Influenza pandemics have many effects on people, health care services and countries. The pandemic spread of Influenza viruses is characterized by a high attack rate and an increased level of mortality particularly in young adults. Therefore, it necessary to understand Influenza viruses that cause pandemics and what strategies can be used for surveillance, mitigation and control.

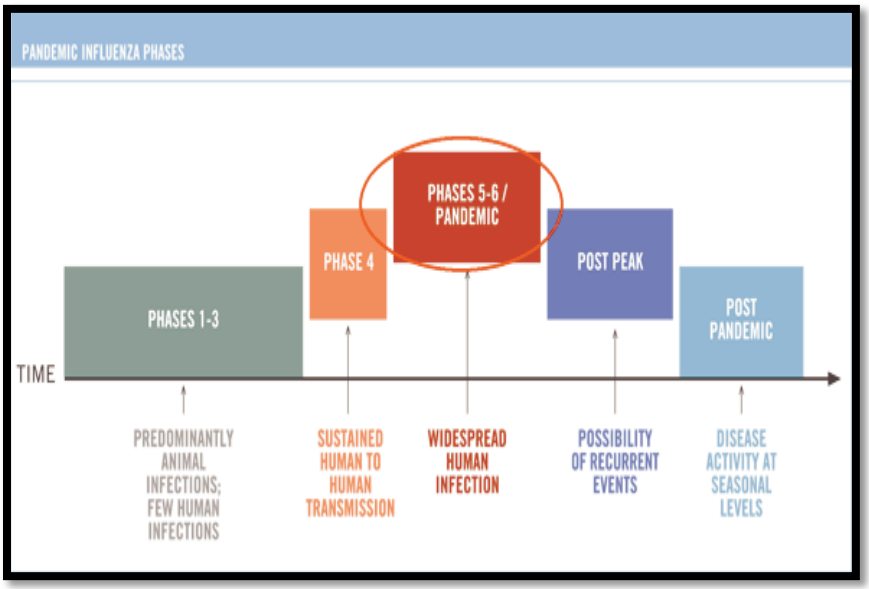



**Fig 2:** **"**Current WHO phase of pandemic alert," accessed on May 4, 2009.

The first case of the flu in India was found on the Hyderabad airport on 13 May, when a man traveling from US to India was found H1N1 positive. Subsequently, more confirmed cases were reported and as the rate of transmission of the flu increased in the beginning of August, with the first death due to swine flu in India in Pune, panic began to spread. As of 24 May 2010, 10193 cases of swine flu have been confirmed with 1035 deaths. (Wikipedia) For early diagnosis and detection of H1N1virus, PCR testing is highly sensitive (lower limit of detection, 1–10 infectious units). Real-time PCR is the test of choice for Influenza A H1N1 2009. It is more rapid and sensitive than cell culture. (Al-Muharrmi; 2010) as the continuous evolution of Influenza genomes together with reassortment events pose challenges to the effective monitoring of Influenza viruses in circulation.

## II. REVIEW OF LITERATURE

Swine flu, also called Hog or Pig Flu, is an infection caused by any one of the several types of Swine Influenza Virus (SIV) which is common throughout pig population worldwide. The term "Influenza" derived from Italianword" Influence" was coined **in 1357 A D**as the disease was thought to be caused by Influence of stars. Influenza pandemics are believed to have occurred at unpredictable intervals for many centuries. (Mir *et al*; 2009) Influenza as a disease of pigs was first recognized during the Spanish Influenza pandemic of 1918–1919. Veterinarian J. S. Koen was the first to describe the illness, observing frequent outbreaks of Influenza in families followed immediately by illness in their swine herds, and vice versa . Influenza virus was first isolated from pigs in 1930 by Shope and Lewis, with the virus isolated from humans several years later. The first isolation of a swine Influenza virus from a human occurred in 1974, confirming speculation that swine origin Influenza viruses could infect humans.(Myers *et al*; 2007)

**2.1History:**

The virus responsible for human epidemic Influenza was first isolated 50 years ago by laboratory infection of ferrets with human nasal washings (Smith *et al*; 1933). This isolation was the culmination of 15 years of research to find the causative agent of the Influenza pandemic of 1918, which in the space of 4 months resulted in 20 million deaths, and since when epidemic Influenza has remained the most serious unconquered acute threat to human health (Grist, 1979)

Global pandemics have been observed for several hundred years. The best documented pandemic occurred in**1918 (A (H1N1), Spanish flu**). It was estimated to have infected 50% of the world's population, with an estimated mortality of 40–50 million (mortality rate of 2–2.5%). The attack and mortality rates were highest among healthy adults (20–40 years old). The second was in **1957 (A(H2N2), Asian flu)** which affected around 40–50% of people during two waves, with a mortality rate of 1 in 4000 and the total death toll probably exceeding 1 million. The third was in **1968 (A (H3N2), Hong Kong flu)** with similar morbidity and mortality to Asian flu. Aspirin use which is known to cause hyperventilation and pulmonary oedema in high doses was the major factor in the high death rate from Spanish flu. Other possible factors could be the unavailability of antibiotics which were not yet discovered to treat bacterial super infection; primitive infection control practices and the destruction of health care facilities as a result of World War I.

On April 15–17, 2009, the Centers for Disease Control and Prevention (CDC) confirmed the first two cases of human infection with the pandemic H1N1 virus in San Diego, California(Rio *et al*;2010). By August 2009, the cumulative number of infections in the United States alone was estimated to be at least 1 million. However, there were only 556 confirmed deaths, i.e. the mortality rate was only 0.056 %.( Al-Muharrmi; 2010)

The outbreak of swine InfluenzaA (H1N1) evolved so rapidly that as on 29 April 2009, nine countries officially reported with confirmed cases of swine Influenza A/H1N1infection. Of these, Mexico, United State, Austria, Canada, Germany, Israel, New Zealand, Spain and the United Kingdom have reported laboratory confirmed human cases and deaths due to rapidly progressive pneumonia, respiratory failure and acute respiratory distress syndrome (ARDS). World Health Organization (WHO) declared ever high stages on its "pandemic" scale- alert 6,designating the Influenza H1N1 2009 a potential threat to worldwide health and declared the outbreak as Public Health Emergency of International Concern (PHEIC).Then in India total confirmed cases and total deaths crossed to a level were the threat of a full blown epidemic is very real (20-21).(Mir *et al*; 2009)

**2.2 Types of Influenza Virus:**

Nucleoprotein and matrix are used to classify Influenza viruses as Types A, B and C.

**2.2.1Influenza Type A:**

2.1 Basic morphology:

The Influenza A virion is

- A globular particle (about 100 nm in diameter).
- Sheathed in a lipid bilayer (derived from the plasma membrane of its host).
- Studded in the lipid bilayer are two integral membrane protein

- (a). some 500 molecules of Hemagglutinin ("H") and
- (b). some 100 molecules of Neuraminidase ("N").
- Within the lipid bilayer is
- Some 3000 species of matrix protein.
- 8 pieces of RNA.
- Each of the 8 RNA molecule is associated with many copies of a Nucleoprotein.
- Several molecules of the three subunits of its RNA polymerase, some "non structural" protein molecules of uncertain function.

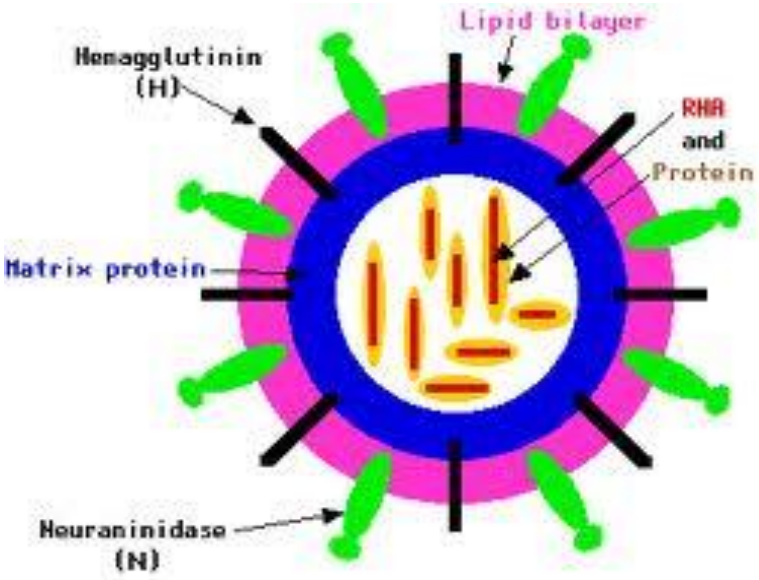



**Fig.** structure of InfluenzaA virus

2.1.1.1**) Diagrammatic representation of the morphology of an Influenza virion**

The virion is generally rounded but may be long and filamentous. A single stranded RNA genome is closely associated with a helical Nucleoprotein (NP), and present in eight separate segments of RiboNucleoprotein (RNP) each of which has to be present for successful replication. The segmented is enclosed within an outer lipoprotein envelope. An antigenic protein called the matrix protein (MP1) lines the inside of the envelope and is chemically bound to the RNP. The envelope carries two types of Protruding spike. One is box shaped protein called the Neuraminidase (NA), of which there are nine major antigenic types, and which has enzymatic properties. The other type of envelope spike is a trimeric protein called Hemagglutinin (HA) of which there are 13 major antigenic types. The Hemagglutinin functions during the attachment of the virus particle to the cell membrane, and can combine with specific receptor on a variety of cell including red blood cells. The lipoprotein envelope makes the virion rather labile- susceptible to heat, drying, detergent and solvents.

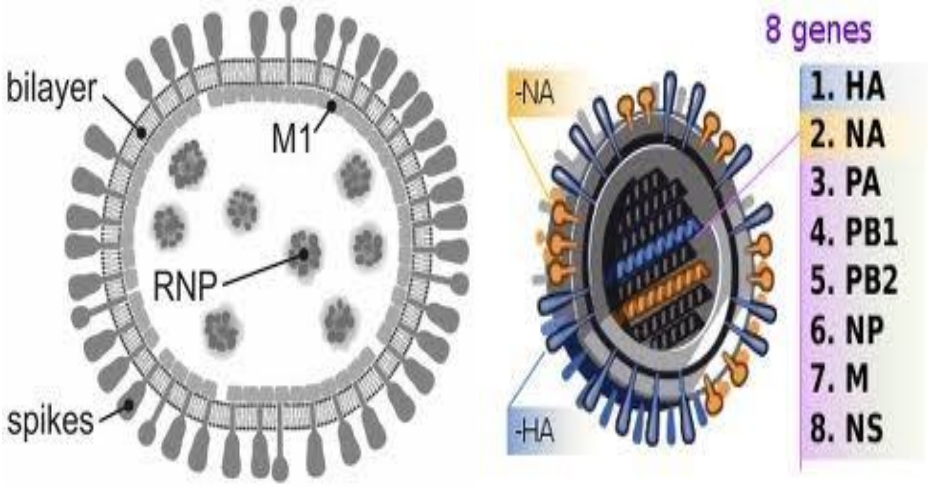



**Fig. (a**)Basic morphology of Influenza virus.(b) Showing 8 separate segment of RNP

2.1.1.2) **The genes of InfluenzaA** virus

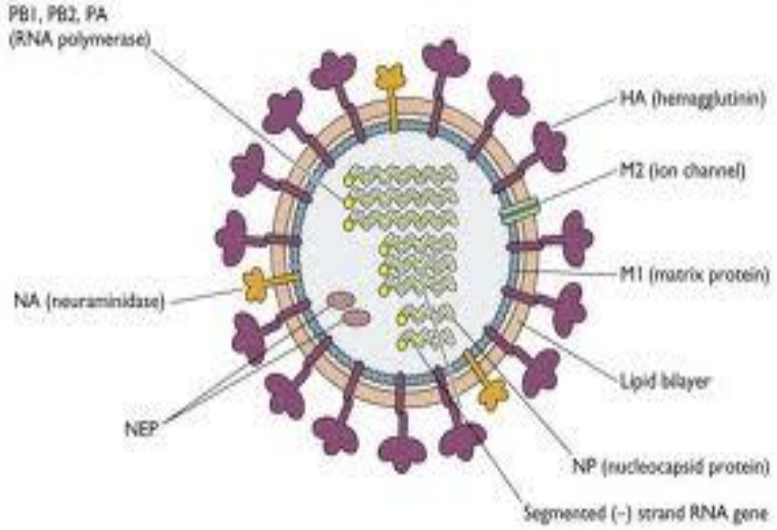



Fig. structures of InfluenzaA virus.

The 8 RNA molecules:

1. The HA gene: - it encodes the hemagglutinin. 3 distinct hemagglutinin, H1, H2, H3, are found in human infections; 13 others have been found in animal flu viruses.
2. The NA gene: - it encodes the neuraminidase. 2 different neuraminidases (N1 and N2) have been found in human viruses; 7 others in other animal.
3. The NP gene encodes the nucleoprotein. Influenza A, B, and C viruses have different nucleoprotein.
4. The M gene encodes two proteins (using different reading frame of the RNA): a matrix protein (M1-shown in blue) and anion channel (M2) spanning the lipid bilayer.
5. The NS gene encodes two different non-structural proteins (also by using different reading frames). These are found in cytosol of infected cell but not within the virion itself.
6. One RNA molecule (PA, PB1, PB2) for each of the 3 subunits of the RNA polymerase.

**2.1.1.3) The Disease**

**The** Influenza virus invades cells of the respiratory passages.

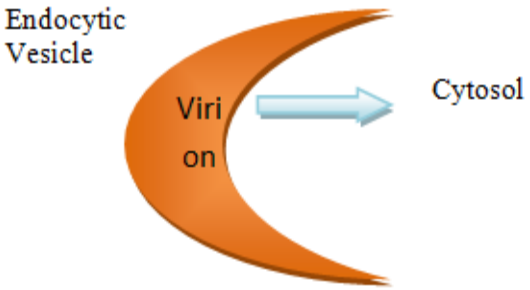



**Fig**. Mechanism of virus invasion.

- Its hemagglutinin molecule bind to sialic acid residues on the glycoprotein's exposed at the surface of epithelial cells of the host respiratory system.
- The virus is engulfed by receptor mediated endocytosis.
- The drop in the pH of the endosome (endocytic vesicle) produces a change in the structure of viral hemagglutinin enabling it to fuse the viral membrane with the vesicle membrane.
- This exposes the contents of virus to the cytosol.
- The RNA enters the nucleus of the cell where fresh copies are made.
- These return to cytosol where some serve as messenger RNA molecule to be translated into the proteins of fresh virus particles.

- Fresh virus buds off from the plasma membrane of the cell (aided by the M2 protein) thus spreading the infection to new cells.

**2.2 Influenza Type B:**

Influenza B viruses are normally found only in humans. Unlike Influenza A viruses, these viruses are not classified according to subtype. Although Influenza type B viruses can cause human epidemics, they have not caused pandemics.

**2.3Influenza Type C**:

Influenza type C viruses cause mild illness in humans and do not cause epidemics or pandemics. These viruses are not classified according to subtype.

**2.3.1Structure of Swine Influenza (H1N1) Virus:**

Influenza viruses are enveloped, segmented, single- stranded, negative- sense RNA viruses belonging to the Orthomyxoviridae family. Influenza viruses contain eight RNA genes that code for eight proteins–internal and external structural proteins, RNA polymerase, and non structural proteins.(Gramer *et. al*; 2005)

Each gene segment contains a coding region that encodes one or two viral proteins; three segments (1, 2 and 3) encode proteins that form the viral polymerase complex: polymerase basic protein 2 (PB2), PB1 and polymerase acidic protein (PA), respectively. Two segments (4 and 6) encode surface envelope glycoprotein's that function as viral antigens, HA and NA, respectively. Segment 5 encodes NP. Segment7 encodes two proteins, the matrix protein M1 and M2.The smallest segment 8 encodes two non- structural proteinsNS1 and NS2.Three phylogenetically and antigenically distinct viral subtypes, A, B and C, are circulating globally among human populations, and subtype A Influenza viruses have exhibited the greatest genetic diversity, infected the wide strange of host species, and caused the vast majority of severe disease in humans. The Influenza A viruses are further subdivided by antigenic characterization of the surface glycoprotein's HA and NA; so far, 16 HA subtypes(H1-H16) and nine NA subtypes (N1-N9) are known (Mayeda *et. al*;2010).These two proteins are involved in cell attachment and release from cells, and are also major targets for the immune response.2,20,131 Wild birds carry most of the known Hemagglutinin and Neuraminidase antigens, but some, such as H14 and H15, are uncommon and seem to occur only in limited geographic areas. Only limited subtypes are found in each species of mammal. Influenza A viruses are also classified into strains. Strains of Influenza viruses are described by their type, host, place of first isolation, strain number (if any), year of isolation, and antigenic subtype.1,3 [e.g., the prototype strain of the H7N7 subtype of equine Influenza virus, first isolated in Czechoslovakia in 1956, is A/eq/Prague/56 (H7N7).] For human strains, the host is omitted.

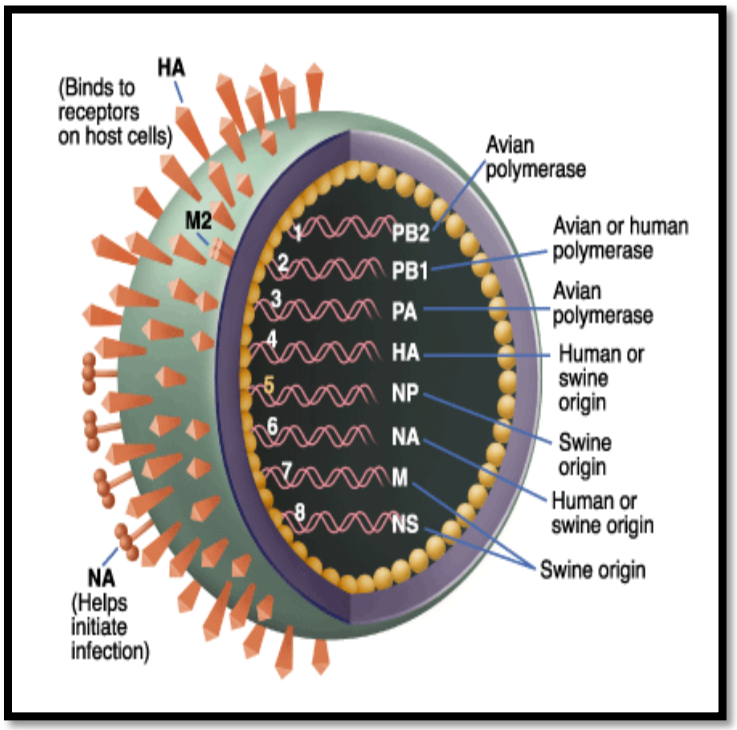



**Fig 3:** Structure of Influenza virus

**2.3.2)Evolution of Influenza Virus:**

Influenza A virus evolution is considered to be pre dominantly driven by two mechanisms known as **antigenic drift** and **antigenic shift**. Influenza viruses are changing by antigenic drift all the time, but antigenic shift happens only occasionally. Influenza types A viruses undergo both kinds of changes; Influenza type B viruses change only by the more gradual process of antigenic drift.

**2.3.3) Antigenic Drift:**

Antigenic drift occurs by random mutation and single amino acid substitution in the HA and NA proteins during viral replication. The change is gradual and part of the normal drift seen with SIV. For the HA gene of Influenza viruses, a mutation occurs at the rate of one mutation in every 100 replicated genes. This rate is sufficiently high enough to create several antigenic variants each year. As in all RNA viruses, mutations in Influenza occur frequently because the virus' RNA polymerase has no proofreading mechanism, providing a strong source of mutations. Mutations in the surface proteins allow the virus to elude some host immunity.

**2.3.4) Antigenic shift:**

Antigenic shift is an abrupt, major change in the Influenza A viruses by which two or more different strains of a virus, or strains of two or more different viruses, combine to form a new subtype having a mixture of the surface antigens of the two or more original strain resulting in a new Influenza virus that can infect humans and has a Hemagglutinin protein or Hemagglutinin and Neuraminidase protein combination that has not been seen in humans for many years. When two different strains of Influenza infect the same cell simultaneously, their protein capsids and lipid envelopes are removed, exposing their RNA, which is then transcribed to mRNA. The host cell then forms new viruses that combine their antigens; for example, H3N2 and H5N1 can form H5N2 this way.

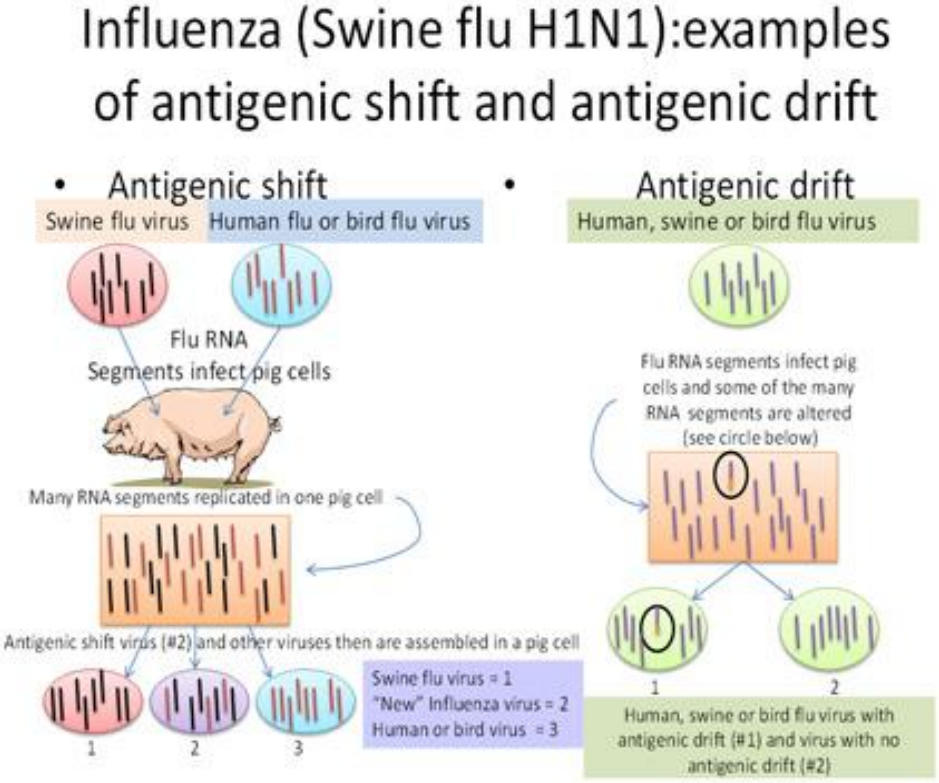



**Fig :** Diagram of Antigenic Drift and Shift

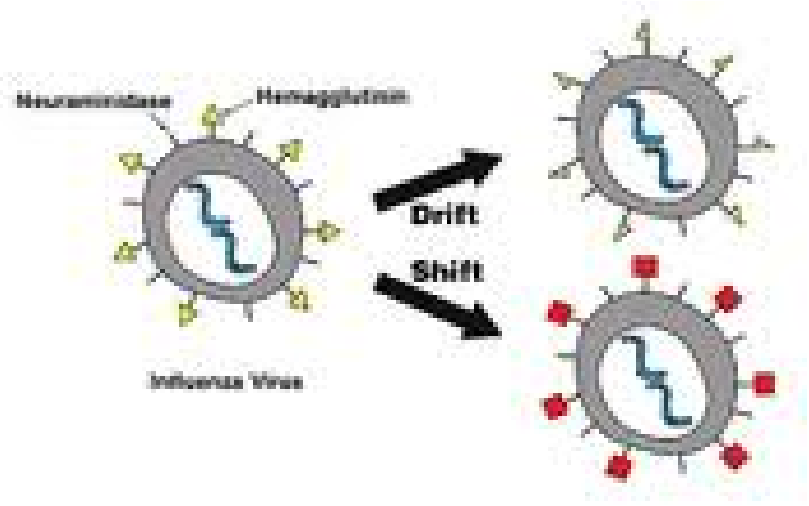



**Fig 4:** Diagram of Antigenic Drift and Shift

**2.3.5)_Transmission between pigs**

Influenza is quite common in pigs, with about half of breeding pigs having been exposed to the virus in the US. Antibodies to the virus are also common in pigs in other countries.

The main route of transmission is through direct contact between infected and uninfected animals. These close contacts are particularly common during animal transport. Intensive farming may also increase the risk of transmission, as the pigs are raised in very close proximity to each other. The direct transfer of the virus probably occurs either by pigs touching noses, or through dried mucus. Airborne transmissions through the aerosols produced by pigs coughing or sneezing are also an important means of infection. The virus usually spreads quickly through a herd, infecting all the pigs within just a few days. Transmission may also occur through wild animals, such as wild boar, which can spread the disease between farms.

**Transmission to humans**

People who work with poultry and swine, especially people with intense exposures, are at increased risk of zoonotic infection with Influenza virus endemic in these animals, and constitute a population of human hosts in which zoonosis and reassortment can co- occur. Vaccination of these workers against Influenza and surveillance for new Influenza strains among this population may therefore be an important public health measure. Transmission of Influenza from swine to humans who work with swine was documented in a small surveillance study performed in 2004 at the University of Iowa. This study among others forms the basis of a recommendation that people whose jobs involve handling poultry and swine be the focus of increased public health surveillance. Other professions at particular risk of infection are veterinarians and meat processing workers, although the risk of infection for both of these groups is lower than that of farm workers.

**2.3.6) Pathogenesis & Replication**

The predominant way in which Influenza is transmitted is from person to person by aerosols and droplets. Influenza then enters the host through the respiratory tract. In a human lung there are about 300 million terminal sacs, called alveoli, Small droplets with a diameter of approximately 1 to 4 µm precipitate in the small airways. Much larger particles are either not able to enter the respiratory system or are deposited in the upper respiratory tract. The respiratory tract is covered with a mucociliary layer consisting of ciliated cells, mucus-secreting cells and glands. Foreign particles in the nasal cavity or upper respiratory tract are trapped in mucus, carried back to the throat, and swallowed. From the lower respiratory tract foreign particles are brought up by the ciliary action of epithelial cells. In the alveoli that lack cilia or mucus, macrophages are responsible for destroying particles.

**Binding to the host cells**

In Influenza infection, the receptor binding site of viral Hemagglutinin (HA) is required for binding to galactose bound sialic acid on the surface of host cells (Weis 1988). Certain areas of the binding site of HA are highly conserved between subtypes of the Influenza virus (Daniels 1984). Hosts may prevent the attachment by several mechanisms: (1) specific immune response and secretion of specific IgA antibodies, (2) unspecific mechanisms, such as mucociliary clearance or production of mucoproteins that able to bind to viral

Hemagglutinin, and (3) genetic diversification of the host receptor (sialic acid), which is highly conserved in the same species, but differs between avian and human receptors (Matrosovich ;2000). The virulence of the Influenza virus depends on the compatibility of Neuraminidase with Hemagglutinin. A virulent virus which has undergone mutations in the Hemagglutinin needs compensatory mutations in the Neuraminidase to maintain its virulence (Baigent& McCauley 2003, Hulse 2004). Once the cell membrane and the virus have been closely juxtaposed by virus- receptor interaction, the complex is endocytosed. Importing H+ ions into the late endocytic vesicles as a physiologic event then acidifies the interior. Upon acidification, the viral HA undergoes a conformational rearrangement that produces a fusiogenic protein.

The loop region of the HA becomes a coiled coil eventually bringing the viral and endosomal membranes closer so that fusion can occur. To allow release of viral RNA into the cytoplasm, the H+ ions in the acidic endosome are pumped into the virion interior by the M2 ion channel. As a result, viral RNA dissociates from M1 by disrupting the low pH- sensitive interaction between the M1 and Ribonuclein complex after fusion of the viral and endosomal membranes. The viral RNA is then imported in an ATP- dependent manner into the nucleus for transcription and translation (Flint 2004).Once Influenza has efficiently infected respiratory epithelial cells, replication occurs within hours and numerous virions are produced. Infectious particles are preferentially released from the apical plasma membrane of epithelial cells into the airways by a process called budding. This favors the swift spread of the virus within the lungs due to the rapid infection of neighboring cells. Alterations in the HA cleavage site by naturally occurring mutants can dramatically influence the tropism and pathogenicity of Influenza. As a result, it can be recognized by other cellular proteases. This leads to higher local concentrations of this ubiquitous protease precursor and thus to increased cleavage of the HA.

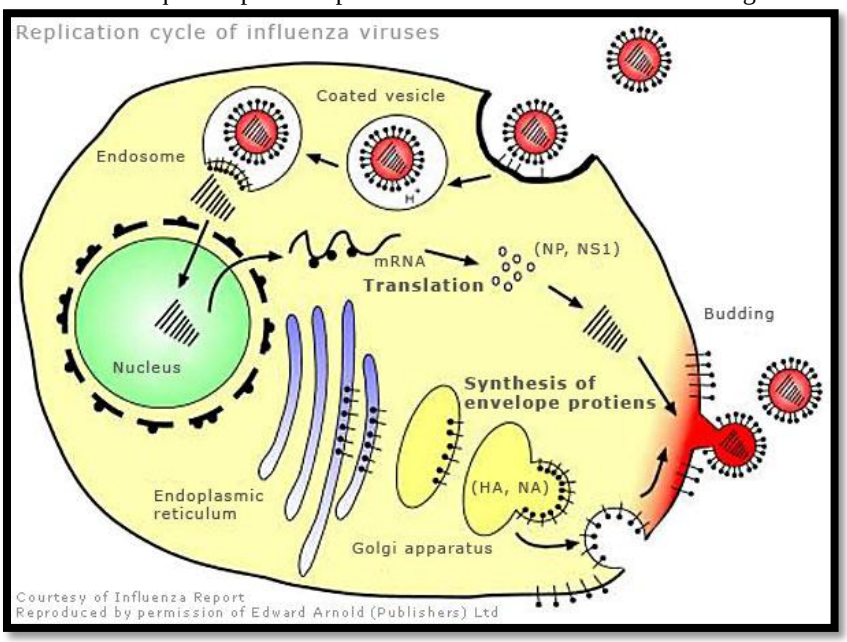



**Fig 5:** Replication Cycle of InfluenzaA virus

**2.4) Immunology**

Influenza causes an acute infection of the host and initiates a cascade of immune reactions activating almost all parts of the immune defense system. Most of the initial innate response, including cytokine release (IFNα/β), influx of neutrophil granulocytes or natural killer cells (Mandelboim 2001, Achdount 2003), and cell activation, is responsible for the acute onset of the clinical symptoms. Innate immunity is an essential prerequisite for the adaptive immune response, firstly, to limit the initial viral replication and antigen load, and secondly, because the antigen- specific lymphocytes of the adaptive immune response are activated by co-stimulatory molecules that are induced on cells of the innate immune system during their interaction with viruses. Influenza viruses, however, encode in the non- structural protein 1 (NS1) mechanisms to evade and antagonize the IFN α/β response. NS1 is likely to sequester viral dsRNA which prevents recognition of this dangerous molecule by cellular sensors which would otherwise trigger IFN α/β release (Garcia- Sastre 1998, Garcia- Sastre 2005). The adaptive immune response requires some days to be effective but then helps to contain the viral spread, to eradicate the virus, and finally to establish a memory response resulting in a long-lived resistance to re-infection with homologous virus. Cross-protection within a subtype of Influenza has only rarely been observed and infections essentially induce no protection across subtypes or between types A and B (Treanor 2005). Influenza infection induces both systemic and local antibody (humoral immunity), as well as

cytotoxic T cell responses (cellular immunity), each of which is important in recovery from acute infection and resistance to reinfection.

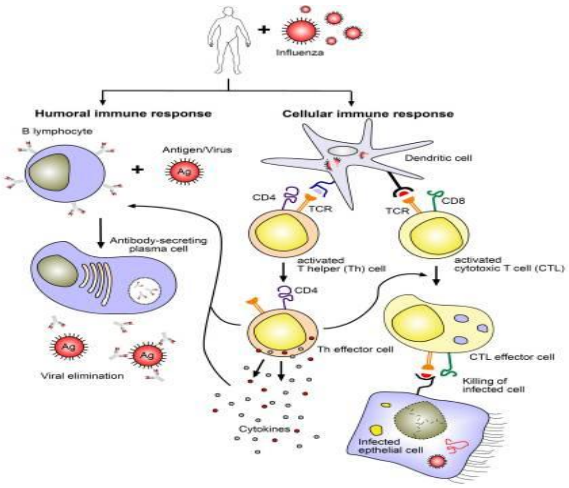



**Figure6:** The humoral and cell-mediated immune response to Influenza virus infection.

The humoral branch of the immune system comprises B-lymphocytes (left), which after interaction with Influenza differentiate into antibody-secreting plasma cells. The cellular response (right) starts with antigen presentation via MHC I (black) and II (blue) molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8). These cells gain effectors cell function to either help directly, release cytokines, or mediate cytotoxicity following recognition of antigen (Adapted from Flint 2004). Not shown is the formation of a cellular memory immune response and the various forms of innate immunity induced by Influenza.

**2.5) Signs and Symptoms:**

- Fever and extreme coldness (chills shivering, shaking (rigor))
- Coughing, sneezing.
- Nasal congestion
- Difficulty breathing and reduced appetite(Kothalwala*et al*;2006)
- Body aches, especially joints and throat
- Fatigue, weight loss.
- Headache
- Irritated, watering eyes
- Reddened eyes, skin (especially face), mouth, throat and nose
- In children, gastrointestinal symptoms such as diarrhea and abdominal pain,[Richards *et al*; 2005] (may be severe in children with Influenza B)[Karr *et al*; 1975]

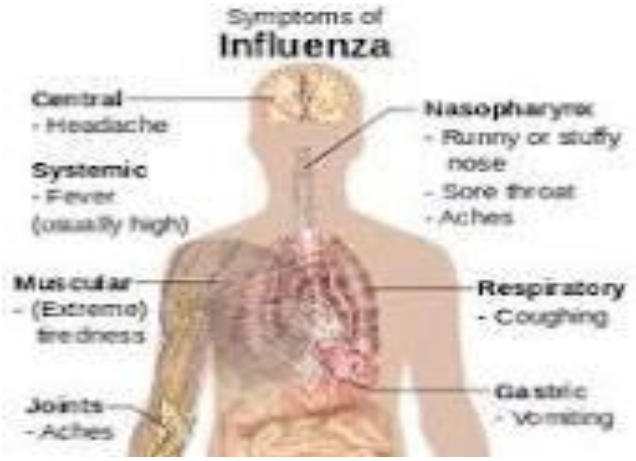



**Fig 7:** Symptoms of Influenza virus

**2.6) Diagnosis:**

InfluenzaA H1N1 2009 virus can be detected in respiratory specimens by different tests. These tests differ in their sensitivity, specificity and ability to distinguish between InfluenzaA subtypes (e.g. 2009 H1N1 versus seasonal H1N1 versus seasonal H3N2 viruses).

**2.6.1) Rapid Influenza diagnostic tests (RIDTs)**, have variable sensitivities and specificities, some experts having reported sensitivity of 47%, and specificity of 86%.48 Others have reported sensitivity of 51%, and specificity of 99% (Faix*et al*;2009).

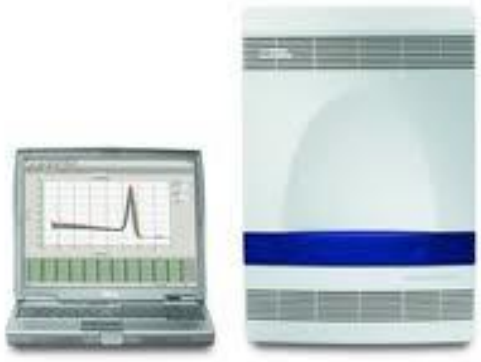
**2.6.2) Direct immunofluorescence (DIF)** has variable sensitivities (47–93%), but high specificity ≥96 %(Faix et al; 2009). Some reports claim that the DIF has a sensitivity of 93%, specificity of 97%, positive predictive value of 95% and negative predictive value of 96%(Pollock et al;2009).

**2.6.3) Viral culture** was the gold standard for Influenza virus testing; however, it is only 88.9% sensitive for InfluenzaA H1N1 2009(Ginocchio*et al*; 2009). Therefore, a negative viral culture does not exclude infection with InfluenzaA H1N1 2009(CDC; 2009). Some researchers have described detection of the virus using microarray techniques (Lu et al; 2009)

**2.6.4) PCR testing** is highly sensitive (lower limit of detection, 1–10 infectious units)(Petric *etal*; 2006). Real-time PCR is the test of choice for InfluenzaA H1N1 2009(WHO; 2009). It is more rapid and sensitive than cell culture. However, PCR is expensive and labour intensive; therefore, it is impractical to investigate all affected patients because of the large number of people infected (Al-Muharrmi; 2010)

**2.6.5) RT-PCR for Diagnosis of H1N1:**

The most Powerful DNA amplification technology known as on date In molecular biology first described by (Higuchi, 1992; Higuchi, 1993). Real- Time Polymerase Chain Reaction, also called quantitative Real Time Polymerase Chain Reaction (qPCR) or kinetic polymerase chain reaction, is a laboratory technique based on the polymerase chain reaction, which is used to amplify and simultaneously quantify a targeted DNA molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of a specific sequence in a DNA sample. The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is quantified as it accumulates in the reaction in real time after each amplification cycle. Two common methods of quantification are the use of fluorescent dyes that intercalate with double- stranded DNA, and modified DNA oligonucleotide probes that fluoresce when hybridized with a complementary DNA.



Real Time PCR

**2.6.6) Principle of RT-PCR:**

Real time quantitative PCR uses fluorophores in order to detect levels of gene expression Cells in all organisms regulate their cellular activities by activating or deactivating the expression of their genes. Gene expression is usually directly proportional to the number of copies of messenger RNA (mRNA) of a particular gene in a cell or tissue. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The polymerase chain reaction is a common method for amplifying DNA; for mRNA- based PCR the RNA sample is first reverse transcribed to cDNA with reverse transcriptase. Development of PCR technologies based on reverse transcription and fluorophores permits measurement of DNA amplification during PCR in real time, i.e., the amplified product is measured at each

PCR cycle. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples, or mRNA copy number. Real-time PCR can also be applied to the detection and quantification of DNA in samples to determine the presence and abundance of a particular DNA sequence in these samples.

Real- time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle (i.e. in real time) as opposed to the endpoint detection. The real-time PCR system is based on the detection and quantitation of a fluorescent reporter (Lee, 1993; Livak, 1995). This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles indicates the detection of accumulated PCR product.

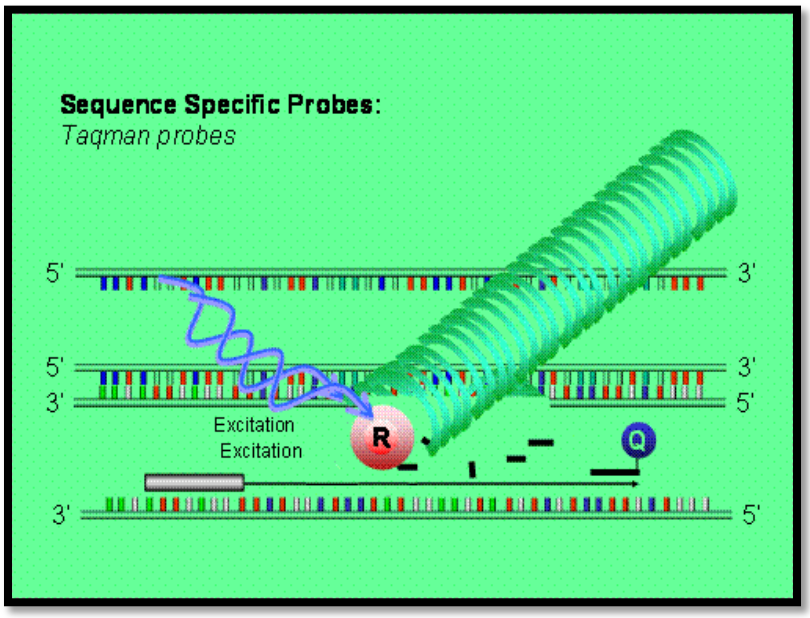
**2.6.7) Various Probe formats**

There are three main fluorescence-monitoring systems for DNA amplification 7 (Wittwer, 1997a):

**(1) Hydrolysis probes**

Hydrolysis probes include TaqMan probes (Heid, 1996), molecular beacons (Mhlanga, 2001; Vet, 2002; Abravaya, 2003; Tan, 2004; Vet &Marras, 2005) and scorpions. They use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences in cDNA samples (see also 19 Svanvik, 2000 for light- up probes).

**TaqMan probes** are oligonucleotides longer than the primers (20-30 bases long with a Tm value of $10^{o}$C higher) that contain a fluorescent dye usually on the 5' base, and a quenching dye (usually TAMRA) typically on the 3' base (TaqMan MGB probes have a non- fluorescent quencher and minor groove binder at the 3' end). When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing. Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the 5' end of probe which contains the reporter dye 22 (Holland, 1991). This ends the activity of quencher and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the origin of the detected fluorescence is specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there is no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage. Well-designed TaqMan probes require very little optimization



**2.7) Treatment:**

The two classes of antiviral drugs used against Influenza are Neuraminidase inhibitors and M2 protein inhibitors (adamantane derivatives). Neuraminidase inhibitors are currently preferred for flu virus infections since they are less toxic and more effective. As pregnant women seem to be more severely affected than the general population by the 2009 H1N1 Influenza virus, prompt treatment with anti-Influenza drugs has been recommended.[107 Jamieson *et al;*2009]

**2.7.1) Neuraminidase inhibitors**

Antiviral drugs such as oseltamivir (trade name Tamiflu) and zanamivir (trade name Relenza) are Neuraminidase inhibitors that are designed to halt the spread of the virus in the body.[108Moscona;2005] These drugs are often effective against both Influenza A and B.[109Stephensons et al 1999] The Cochrane Collaboration reviewed these drugs and concluded that they reduce symptoms and complications.[110Jefferson et al;2006] Different strains of Influenza viruses have differing degrees of resistance against these antivirals, and it is impossible to predict what degree of resistance a future pandemic strain might have.

**2.7.2) M2 inhibitors (adamantanes)**

The antiviral drugs amantadine and rimantadine block a viral ion channel (M2 protein) and prevent the virus from infecting cells.[43Pinto et al;2006] These drugs are sometimes effective against Influenza A if given early in the infection but are always ineffective against Influenza B because B viruses do not possess M2 molecules. Measured resistance to amantadine and rimantadine in American isolates of H3N2has increased to 91% in 2005. [112CDC; 2006] This high level of resistance may be due to the easy availability of amantadines as part of over- the- counter cold remedies in countries such as China and Russia,[113Bright et al;2006]and their use to prevent outbreaks of Influenza in farmed poultry.(Wikipedia)

**2.8Vaccination**

There are two types of vaccine:

**2.8.1) Trivalent Inactivated Vaccine (Tiv)**

The trivalent inactivated vaccine (TIV) is an inactivated preparation of egg- grown virus and is given by injection. Only certain formulations of the vaccine are FDA certified for young children – the annual ACIP recommendations (see below) give details. Protection is via IgG antibodies.

**2.8.2) Live Attenuated Influenza Virus Vaccine (Laiv)**

The live, attenuated Influenza virus (LAIV - marketed as FluMist) vaccine (see Genetics Lecture) is prepared from egg- grown virus. It is approved for healthy (those not at risk for complications from Influenza infection), non- pregnant individuals 2 to 49 years old but should not be given to children under 5 years of age who have possible reactive airways disease (for example, a history of recurrent wheezing). It is given nasally and should provide mucosal, humoral and cell- mediated immunity. It is contraindicated for children and adolescents on any therapy containing aspirin due to the potential risk of Reye's syndrome since the virus is a live virus.

Both Influenza vaccines are formulated annually using the types and strains of Influenza predicted to be the major problems for that year (the predictions are based on worldwide monitoring of Influenza). The vaccines are multivalent, the current ones are trivalent and have two strains of Influenza A and one of Influenza B. Vaccination needs to be given every year because the most effective strains for the vaccine will change due to drift and/or shift. The vaccines are usually given in the Fall , once the strains to be used for the Influenza season have been determined in the earlier part of the year. By giving the vaccine in the fall, protection should be high at the time the Influenza season peaks. Since both vaccines are grown in eggs, they are contraindicated for those allergic to eggs.

**2.9) Prevention:**

Take these everyday steps to protect your health:

- Cover your nose and mouth with a tissue when you cough or sneeze. Throw the tissue in the trash after you use it.
- Wash your hand often with soap or water, especially after you cough or sneeze. You can also use an alcohol based hand cleaner.
- Avoid touching your eyes, nose or mouth. Germs spread this way.
- Try to avoid close contact with sick people.

- Stay home if you are sick until at least 24 hours after you no longer have fever ($100^oF$ or $37.8^oC$) or signs of fever (without the use of fever reducing medicine, such as Tylenol)
- While sick, limit contact with others as much as possible keep from infecting them.

## III. AIMS AND OBJECTIVES

**Aim:** Our Aim of the study is "To analyze age wise positivity rate of H1N1 using RT- PCR in the year 2011- 2012"

**Objective:** To achieve the aim "To analyze the positivity rate of H1N1 using RT- PCR in the year 2011- 2012" following techniques were used-

- Sample Collection
- Viral RNA Isolation
- Diagnosis using RT- PCR

## IV. MATERIALS AND METHODS

**4.1) Sample Collection:**

- Samples are collected from various NCR regions and then they carried to molecular testing laboratory for further processing and analysis.
- Samples should be taken from the nasopharynx (a nasopharyngeal swab), nasopharyngeal aspirates, throat swabs and transbronchial aspirates.
- Swab specimens should be collected using swabs with a synthetic tip (e.g. polyester or Dacron®), but not calcium alginate or cotton tips; the shaft should be made of aluminum or plastic, but not of wood.
- Specimens should be placed into sterile viral transport media.

**4.1) Materials for specimen collection:**

**4.1.1) Transport Media**

1. B D Viral transport media

**OR**

2. In house viral transport medium

***(A)*Medium 199**

Tissue culture medium 199 containing 0.5% bovine albumin fraction VPenicillin G (2 X 106 U/liter), Streptomycin 200 mg/liter, polymyxin(2 x 106 U/liter), gentamicin (250 mg/liter), nystatin (0.5 X 106 U/liter). Ofloxacin HCI (60 mg/liter), and sulfamethoxazole (0.2 g/ liter).

**(B) Broth media**

10g veal infusion broth, 2g of BSA fraction V add to 400ml sterile distilled waterPenicillin G (2 X 106 U/liter), Streptomycin 200 mg/liter, Polymyxin (2 x 106U/liter), Gentamicin (250 mg/liter), nystatin (0.5 X 106 U/liter). Ofloxacin HCI (60mg/liter), and Sulfamethoxazole (0.2 g/ liter).

Sterilize by filtration and distribute in 1.0ml - 2.0ml volumes in

- Screw capped tubes
- Dacron swabs. Calcium alginate is not accepted for the collection of viral specimens
- Tongue depressor

**4.2) UPPER RESPIRATORY TRACT SPECIMENS**

**4.2.1) Method of collecting a throat swab**

1. Hold the tongue down with the depressor. Use a strong light source to locate areas of inflammation in the posterior pharynx and the tonsillar region of the throat behind the uvula.
2. Rub the area back and forth with the swab. Withdraw the swab without touching cheeks, teeth or gums and insert into a screw- cap vial containing viral transport medium.
3. Break off the top part of the stick without touching the tube and tighten the screw capfirmly
4. Label the specimen containers with patient's name type of specimen and date ofcollection
5. Complete the laboratory request form.

**4.2.2) Method of collecting Nasopharyngeal Swabs (per- nasal and post nasal swab)**

1. Seat the patient comfortable, tilt the head back
2. Insert a flexible swab beneath the inferior turbinate of either nostril or leave in place for afew seconds and move the swab upwards into the nasopharyngeal space.
3. Rotate the swab on the nasopharyngeal membrane a few times; slowly withdraw with arotating motion against the mucosal surface of the nostril.
4. Remove the swab carefully and insert it into a screw- cap tube containing transportmedium.
5. Repeat the procedure in the other nostril using a new sterile swab The tip of each swab isput into a vial containing 2- 3 ml of viral transport media (VTM), and the applicator stick isbroken off.
6. Label vial with patient's name, specimen type & date of collection; complete lab requestform.

**4.2.3) Aspirates**

1. Nasopharyngeal secretions are aspirated through catheter connected to a mucus trapand fitted to a vacuum source.
2. The nasal aspirates are collected by introducing a few ml of saline into the nose with asyringe fitted with affine tubing or catheter.
3. The catheter is inserted into a nostril parallel to the palate. Then the vacuum is appliedand the catheter is slowly withdrawn with a rotation motion.
4. Mucus from the other nostril is collected with the same catheter in a similar manner.
5. After mucus has been collected from both nostrils, the catheter is flushed into a screwcap vial with 3 ml viral transport media
6. Label the vial with patient's name type of specimen and date of collection**.**
7. Complete the laboratory request form.

**4.3) SHIPMENT OF SPECIMENS**

The specimen(s) must be shipped immediately, if delay is more than 4 hours of collection thespecimen should be refrigerated and send with ice packs.

**4.4) Packing of Samples**

1. Wrap the primary container (the container in which the specimen is enclosed such as avial) with parafilm or sealing tape around the lid. The container must then be wrapped withenough absorbent material to absorb all of the fluid in the primary container. (Note: If usingpaper towels as absorbent material, use at least one paper towel for each 1.5 ml of fluid).
2. Additional absorbent should be placed around the container to prevent breakage duringtransport.
3. Place the specimen primary container and absorbent wrapping into a sealable plastic bag(the specimen +absorbant+ plastic bag).
4. Place the plastic bag (the specimen +absorbant+ plastic bag) into a secondary closecontainer.

**4.5) Indicate the following on the Laboratory request form**

1. Patient demographics
2. Clinical signs and symptoms
3. Date of onset of illness and date of collection of specimen
4. Type of specimen
5. Travel history and/or contact of known case

**4.6) In Land Transportation of Diagnostic Specimens**

If the sentinel site is located away from the National Laboratory

1. Place the plastic bag (the specimen +absorbant+ plastic bag) into a secure the secondary safety container is showed in the figure below.
2. Place the sample container in a cooler with ice packs to ensure specimen integrity in hot weather during transit from the sentinel site to the national laboratory
3. Send the specimen and the Laboratory Request Form with the a previously trained carrier or driver dedicated to the transportation of specimens

**4.7) Overseas Transportation of Diagnostic Specimens**

1. Place the plastic bag (the specimen +absorbant+ plastic bag) into a secure thesecondary safety container is showed in the figure below with the laboratory request form.
2. Place the sample container in cardboard container with ice packs.
3. Communicate with your national public health authority before referring samples toSatellite and CAREC laboratories.
4. Notify the satellite and CAREC laboratory of the shipment of clinical specimens.

5. Submit specimens to your Satellite Laboratory and CAREC Laboratory Division, through the National Laboratory according CAREC guidelines and the IATA regulations "Diagnostic specimens" UN 3373.

**4.8) Packing and Labeling of Clinical Specimens**

**4.8.1) Transportation of specimens**

Specimens should be sent as "diagnostic specimens" in accordance with the International Air transport Association dangerous goods regulations.

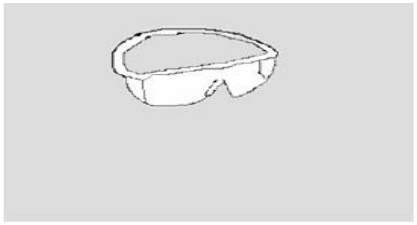
**4.10) Equipments used during Sample Handling:**

**4.10.1) Personal Protection Equipments:**

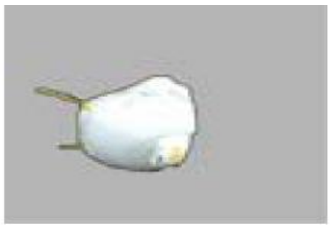
Before initiating processing of samples a fullcomplement of PPE should be worn**.** Thisincludes:

• Gloves (nonsterile),
• Mask (high- efficiency mask) / Three layered surgical mask,
• Long- sleeved cuffed gown,
• Protective eyewear (goggles/visors/face shields),
• Cap (may be used in high risk situations where there may be increasedaerosols),
•Plastic apron if splashing of blood, body fluids, excretions and secretions isanticipated.

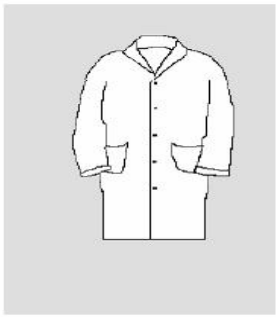
Fig: showing PPE bearing inside and outside the laboratory



Goggles

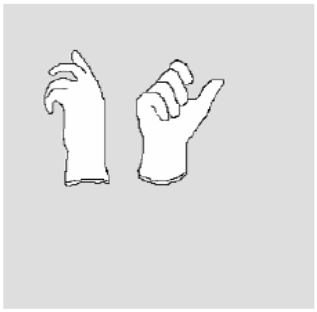


N-95 Mask
OR

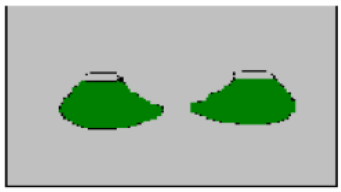


Gown(must for lab work)

Triple layer Mask



Gloves



Shoe covers

**Fig:** Personal Protection Equipment

The PPE should be used in situations were regular work practice requires unavoidable, relatively closed contact with the suspected human case /poultry.

**4.10.2) Correct procedure for applying PPE in the following order:**

1. Follow thorough hand wash
2. Wear the coverall.
3. Wear the goggles/ shoe cover/and head cover in that order.
4. Wear face mask
5. Wear gloves

The masks should be changed after every six to eight hours.

**4.10.3) Remove PPE in the following order:**

• Remove gown (place in rubbish bin).
• Remove gloves (peel from hand and discard into rubbish bin).
• Use alcohol- based hand- rub or wash hands with soap and water.
• Remove cap and face shield (place cap in bin and if reusable place face shield incontainer for decontamination).
• Remove mask- by **grasping elastic behind ears – do not touch front of mask**
• Use alcohol- based hand- rub or wash hands with soap and water.
• Leave the room.
• Once outside room use alcohol hand- rub again or wash hands with soap andwater.

❖ **Used PPE should be handled as waste as per waste management protocol**

**4.11) SAMPLE PROCESSING**:Viral RNA is isolated after sample reaches the laboratory.

**4.11.1) H1N1 RNA ISOLATION**
**QIAamp Viral Mini Kit, or Rneasy Mini Kit (QIAGEN)**
**4.11.2) COMPONENTS OF RNA EXTRACTION KIT (QIAGEN)**

1. Buffer AVL
2. RNA Carrier (Lyophilized )
3. Buffer AW1
4. Buffer AW2
5. Buffer AE
6. Columns
7. Collection Tube

**Important Instructions to be followed**:

1. QIAamp Mini spin coloumns should be stored dry at room temperature (15- 25°C.). Storage at high temp. Should be avoided.
2. All solutions should be stored at room temperature unless otherwise started.
3. Lyophilized carrier RNA can be stored at room temp. (15-25°C) until the expiration date on the kit box.

**4.11.3) INSTRUCTIONS FOR RNA ISOLATION BY QIAGEN RNA ISOLATION KIT**
**a) Reconstitution of the Reagents:**

1) **RNA Carrier : Add 310 µl of Buffer AVEElution Buffer**
2) **Preparation of Buffer AW1: Add 125ml of Ethanol to make final volume to 220 ml.**
3) **Preparation of Buffer AW2 : Add 160 ml of Ethanol to make final volume to 226 ml**

**b) Procedure:**

1. Pipette 560 µl of AVL containing carrier RNA into 1.5 ml Micro Centrifuge Tube.

Add 140 µl samples to AVL (prepared).

2. Mix by pulse vortexing it for 15 sec.
3. Incubate for 10 min at room temperature, ie. 15- 25°C for 10 min.
4. Add 560 µl of ethanol (96- 100%).
5. Pulse vortex and then pulse centrifuge it for 2 min.
6. Add 630 µl of solution (lysis sample) into QIAamp Mini spin column.
7. Centrifuge at 8000 rpm for 1 min.
8. Transfer the column in a fresh collection tube.
9. Add the remaining sample into the QIAamp Mini spin column.
10. Centrifuge at 8000 rpm for 1min.
11. Discard the filtrate and add 500 µl AW1 buffer.
12. Centrifuge at 8000 rpm for 1 min
13. Discard the collection tube and place the column in fresh collection tube.
14. Add 500 µl of AW2.
15. Centrifuge at 14,000 rpm for 3 min (repeat if necessary).
16. Place the column in a fresh Micro Centrifuge Tube and add 60 µl AVE Buffer on the membrane.
17. Incubate for 1 min at RT.
18. Centrifuge at 8000 rpm for 1 min.
19. RNA is isolated.

**c) Master Mix preparation for H1N1:**
**Important work instructions for making H1N1 Master Mix:**

1. Carry all the reagents for H1N1 into Area 1 in a cool box.
2. Always wear sterile powder free gloves while preparing the Master Mix as powder can adversely affect the amplification.
3. Place Master Mix and enzyme in cold rack.
4. Buffer should be aliquoted in 1.5 ml Microcentrifuge Tubes and should be wrapped with aluminium foil, avoid repeated freeze- thaw cycle.
5. Thaw frozen aliquotes of primer and probes (Thawed aliquots of probes may be stored in the dark up to 3 months at 2- 8 C. Do not re- freeze probes).
6. Vortex all primers and probes.
7. Briefly centrifuge all the primers and probes and then place in cold rack.

**Master Mix Composition:**

| S. No | Component | Final vol. for 1X |
|---|---|---|
| 1. | Buffer Mix | 12.5 µl |
| 2. | Enzyme Mix | 1.0 µl |
| 3. | Nuclease Free $H_2O$ | 6.0 µl |
| 4. | Assay Mix | 0.5 µl |
| 5 | **Total** | **20.0 µl** |

The above process is for 1 reaction but to perform for multiple samples, multiply the no. of samples with the above composition.

**Each Sample:**

**RNase P × 2 + 1 (NTC) + 1 (Pipetting error)**
**Inf A × 2 +1 (NTC) + 1 (Pipetting error)**
**Swinf × 2 + 1(NTC) + 1 (Pipetting error)**
**SwiH1 × 2 + 1(NTC) + 1(Pipetting error)**

**d) Primer and probe sets:**

| Primers and probes | Sequence( 5' >3') | Working Concentration |
|---|---|---|
| Inf A Forward | GAC CRA TCC TGT CAC CTC TGA C | 40 µM |
| Inf A Reverse | AGG GCA TTY TGG ACA AAK CGT CTA | 40 µM |
| Inf A Probe | TGC AGT CCT CGC TCA CTG GGC ACG | 10 µM |
| SW Inf A Forward | GCA CGG TCA GCA CTT ATY CTR AG | 40 µM |
| SW Inf A Reverse | GTG RGC TGG GTT TTC ATT TGG TC | 40 µM |
| SW Inf A Probe | CYA CTG CAA GCC CA"T' ACA CAC AAG CAG GCA | 10 µM |
| SW H1 Forward | GTG CTA TAA ACA CCA GCC TYC CA | 40 µM |
| SW H1 Reverse | CGG GAT ATT CCT TAA TCC TGT RGC | 40 µM |
| SW H1 Probe | CA GAA TAT ACA "T"CC RGT CAC AAT TGG ARA A | 10 µM |
| Rnase P Forward | AGA TTT GGA CCT GCG AGC G | 40 µM |
| Rnase P Reverse | GAG CGG CTG TCT CCA CAA GT | 40 µM |
| Rnase P Probe | TTC TGA CCT GAA GGC TCT GCG CG | 10 µM |

**f) Protocol for Operating 7500 FAST REAL TIME PCR SYSTEM**

1. Turn on the computer and 7500 Fast Real Time PCR System.
2. Place the plate containing samples into the machine
3. Go to 7500 software v 2.0.1 on the desktop.
4. Open a new programme by clicking on new experiment
5. Select 7500 Fast (96 wells) instrument to run the experiment.
6. Select Quantitation Standard curve set up.
7. Select ' Taqman (R) reagents to detect the target sequence.
8. Select Standard (2 hours to complete a run) ramp speed to run the instrument.
9. Go to set up.
10. Go to plate set up.
11. First define Targets by clicking 'Add new targets' and define samples by clicking 'Add new samples'.
12. Assign samples according to the order in MMX plate to the plate layout this is done by selecting 4 wells for each samples and assigning samples by clicking the respective sample I.D.
13. Assign targets to the selected wells
14. Select wells for PC then assign these wells as standard for following genes Inf- A, SwinInf- A, SwH1, and Rnaserespectievely.
15. Select wells for NC and assign these wells as NC for following genes Inf- A, SwinInf- A, SwH1, and Rnaserespectievely.
16. Go to run method
17. Set the thermal profile (cycling conditions and no. of cycle)
18. Save the programme as new one
19. Start run
20. Go to temperature plot and watch carefully for sometime.
21. After the run is complete go to analyse and then analyse the amplification plot.

## V. RESULTS AND DISCUSSIONS

The aim of this prospective study was to identify the positivity rate of H1N1 virus in different age group and sex of patient by using Taqman Real Time PCR approach in a selected population of Delhi (NCR) including Jaipur for the year 2011-2012. Samples were collected from different collection centers and the main specimen type taken was throat and nasal swab in VTM (Viral Transport Medium). The total number of sample collected were 324, in which 8 samples showed positive results while the remaining were negative as shown in the table given below:

| S.No. | Patient I.D. | Age/Sex | Inf A | H1N1 |
|---|---|---|---|---|
| 1. | 2075 | 02Y/M | NO | NO |
| 2. | 2076 | 69Y/M | NO | NO |
| 3. | 2077 | 65Y/M | NO | NO |
| 4. | 2078 | 83Y/M | NO | NO |
| 5. | 2079 | 64Y/M | NO | NO |
| 6. | 2080 | 22Y/M | NO | NO |
| 7. | 2081 | 38Y/F | NO | NO |
| 8. | 2082 | 50Y/F | NO | NO |
| 9. | 2083 | 28Y/F | NO | NO |
| 10. | 2084 | 77Y/M | NO | NO |
| 11. | 2085 | 79Y/M | NO | NO |
| 12. | 2086 | 05Y/M | NO | NO |
| 13. | 2087 | 24Y/M | NO | NO |
| 14. | 2088 | 02Y/M | NO | NO |
| 15. | 2089 | 54Y/F | NO | NO |
| 16. | 2090 | 20Y/F | NO | NO |
| 17. | 2091 | 76Y/M | NO | NO |
| 18. | 2092 | 31Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 20. | 2109 | 27Y/M | NO | NO |
| 21. | 2110 | 26Y/M | NO | NO |
| 22. | 2111 | 20Y/M | NO | NO |
| 23. | 2112 | 06Y/F | NO | NO |
| 24. | 2113 | 85Y/M | NO | NO |
| 25. | 2114 | 21Y/M | NO | NO |
| 26. | 2115 | 79Y/F | NO | NO |
| 27. | 2116 | 74Y/M | NO | NO |
| 28. | 2117 | 06 Y/M | NO | NO |
| 29. | 2118 | 25Y/F | NO | NO |
| 30. | 2119 | 82Y/M | NO | NO |
| 31. | 2120 | 55Y/F | NO | NO |
| 32. | 2121 | 28Y/F | NO | NO |
| 33. | 2132 | 76Y/F | NO | NO |
| 34. | 2133 | 30Y/F | NO | NO |
| 35. | 2134 | 21Y/F | NO | NO |
| 36. | 2135 | 67Y/M | YES | NO |
| 37. | 2136 | 50Y/M | YES | NO |
| 38. | 2137 | 76Y/M | NO | NO |
| 39. | 2138 | 58Y/M | NO | NO |
| 40. | 2139 | 58Y/M | NO | NO |
| 41. | 2140 | 28Y/F | NO | NO |
| 42. | 2141 | 66Y/M | NO | NO |
| 43. | 2142 | 54Y/M | NO | NO |
| 44. | 2143 | 19Y/F | NO | NO |
| 45. | 2144 | 02Y/F | NO | NO |
| 46. | 2145 | 26Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 48. | 2152 | 63Y/F | NO | NO |
| 49. | 2153 | 65Y/F | NO | NO |
| 50. | 2154 | 74Y/M | NO | NO |
| 51. | 2156 | 70Y/M | NO | NO |
| 52. | 2157 | 52Y/F | NO | NO |
| 53. | 2158 | 25Y/M | NO | NO |
| 54. | 2159 | 13Y/F | NO | NO |
| 55. | 2160 | 54Y/F | NO | NO |
| 56. | 2161 | 42Y/M | NO | NO |
| 57. | 2162 | 62Y/M | NO | NO |
| 58. | 2163 | 47Y/F | NO | NO |
| 59. | 2164 | 65Y/F | NO | NO |
| 60. | 2165 | 09Y/F | NO | NO |

| | | | | |
|---|---|---|---|---|
| 61. | 2166 | 49Y/F | NO | NO |
| 62. | 2167 | 82Y/M | NO | NO |
| 63. | 2168 | 32Y/F | NO | NO |
| 64. | 2169 | 65Y/M | NO | NO |
| 65. | 2170 | 40Y/F | NO | NO |
| 66. | 2171 | 58Y/M | NO | NO |
| 67. | 2172 | 59Y/F | NO | NO |
| 68. | 2173 | 59Y/F | NO | NO |
| 69. | 2174 | 48Y/M | NO | NO |
| 70. | 2175 | 40Y/M | NO | NO |
| 71. | 2176 | 72Y/M | NO | NO |
| 72. | 2177 | 24Y/F | NO | NO |
| 73. | 2178 | 58Y/M | NO | NO |
| 74. | 2179 | 68Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 76. | 2181 | 48Y/F | NO | NO |
| 77. | 2182 | 44Y/M | NO | NO |
| 78. | 2183 | 17Y/F | NO | NO |
| 79. | 2184 | 42Y/M | NO | NO |
| 80. | 2185 | 60Y/F | NO | NO |
| 81. | 2186 | 28Y/M | NO | NO |
| 82. | 2187 | 42Y/F | YES | YES |
| 83. | 2188 | 57Y/M | NO | NO |
| 84. | 2189 | 50Y/F | NO | NO |
| 85. | 2204 | 76Y/M | NO | NO |
| 86. | 2205 | 66Y/M | NO | NO |
| 87. | 2206 | 58Y/F | NO | NO |
| 88. | 2207 | 72Y/M | NO | NO |
| 89. | 2208 | 66Y/M | NO | NO |
| 90. | 2244 | 80Y/F | NO | NO |
| 91. | 2245 | 20Y/M | NO | NO |
| 92. | 2246 | 76Y/F | NO | NO |
| 93. | 2247 | 22Y/M | NO | NO |
| 94. | 2248 | 60Y/M | NO | NO |
| 95. | 2249 | 36Y/F | NO | NO |
| 96. | 2250 | 29Y/F | NO | NO |
| 97. | 2251 | 51Y/F | NO | NO |
| 98. | 2252 | 39Y/F | NO | NO |
| 99. | 2253 | 24Y/F | NO | NO |
| 100. | 2254 | 53Y/F | NO | NO |
| 101 | 2255 | 50Y/M | NO | NO |
| 102. | 2256 | 70Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 104. | 2258 | 84Y/F | NO | NO |
| 105. | 2259 | 54Y/M | NO | NO |
| 106. | 2260 | 77Y/M | NO | NO |
| 107. | 2261 | 16Y/F | NO | NO |
| 108. | 2262 | 74Y/F | NO | NO |
| 109. | 2263 | 78Y/M | NO | NO |
| 110. | 2264 | 86Y/F | NO | NO |
| 111. | 2265 | 70Y/M | NO | NO |
| 112. | 2266 | 66Y/F | NO | NO |
| 113. | 2267 | 12Y/M | NO | NO |
| 114. | 2268 | 50Y/F | NO | NO |
| 115. | 2269 | 56Y/F | NO | NO |
| 116. | 2270 | 06Y/F | NO | NO |
| 117. | 2271 | 34Y/M | NO | NO |
| 118. | 2272 | 33Y/M | NO | NO |
| 119. | 2273 | 57Y/M | NO | NO |
| 120. | 2274 | 54Y/M | NO | NO |
| 121. | 2275 | 10Y/M | NO | NO |
| 122. | 2276 | 16Y/M | NO | NO |
| 123. | 2277 | 26Y/M | NO | NO |
| 124. | 2278 | 43Y/M | NO | NO |
| 125. | 2279 | 17Y/F | NO | NO |
| 126. | 2280 | 76Y/F | NO | NO |
| 127. | 2281 | 65Y/M | NO | NO |
| 128. | 2282 | 37Y/F | NO | NO |
| 129. | 2283 | 38Y/F | NO | NO |

| | | | | |
|---|---|---|---|---|
| 130. | 2284 | 61Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 132. | 2286 | 16Y/F | NO | NO |
| 133. | 2287 | 60Y/M | NO | NO |
| 134. | 2289 | 68Y/F | NO | NO |
| 135. | 2290 | 28Y/M | NO | NO |
| 136. | 2291 | 12Y/M | NO | NO |
| 137. | 2292 | 52Y/F | NO | NO |
| 138. | 2293 | 82Y/F | NO | NO |
| 139 | 2294 | 26Y/M | NO | NO |
| 140 | 2295 | 37Y/M | NO | NO |
| 141 | 2296 | 48Y/M | NO | NO |
| 142 | 2297 | 56Y/M | NO | NO |
| 143 | 2298 | 72Y/M | YES | NO |
| 144 | 2299 | 46Y/F | NO | NO |
| 145. | 2300 | 45Y/F | NO | NO |
| 146. | 2301 | 17Y/M | NO | NO |
| 147. | 2302 | 27Y/F | NO | NO |
| 148. | 2303 | 32Y/F | NO | NO |
| 149. | 2304 | 40Y/F | NO | NO |
| 150. | 2305 | 50Y/F | NO | NO |
| 151. | 118 | 69Y/M | NO | NO |
| 152. | 124 | 82Y/F | NO | NO |
| 153. | 125 | 70Y/F | NO | NO |
| 154. | 126 | 56Y/M | NO | NO |
| 155. | 127 | 75Y/F | NO | NO |
| 156. | 128 | 76Y/F | NO | NO |
| 157. | 129 | 45Y/F | NO | NO |
| 158. | 130 | 46Y/F | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 160. | 132 | 52Y/F | NO | NO |
| 161. | 133 | 51Y/F | NO | NO |
| 162. | 141 | 46Y/F | NO | NO |
| 163. | 142 | 58Y/F | NO | NO |
| 164. | 143 | 23Y/M | NO | NO |
| 165. | 144 | 44Y/M | NO | NO |
| 166. | 145 | 39Y/F | NO | NO |
| 167. | 146 | 70Y/F | NO | NO |
| 168. | 147 | 57Y/M | YES | NO |
| 169. | 148 | 43Y/M | NO | NO |
| 170. | 149 | 50Y/M | NO | NO |
| 171. | 153 | 54Y/F | NO | NO |
| 172. | 154 | 24Y/M | NO | NO |
| 173. | 155 | 16Y/M | NO | NO |
| 174. | 156 | 22Y/M | NO | NO |
| 175. | 119 | 86Y/M | NO | NO |
| 176. | 120 | 10Y/F | NO | NO |
| 177. | 103 | 53Y/F | NO | NO |
| 178. | 104 | 70Y/M | YES | NO |
| 179. | 105 | 28Y/F | NO | NO |
| 180. | 106 | 74Y/F | NO | NO |
| 181. | 107 | 37Y/F | NO | NO |
| 182. | 108 | 16Y/M | NO | NO |
| 183. | 109 | 58Y/M | NO | NO |
| 184. | 110 | 59Y/M | NO | NO |
| 185. | 111 | 67Y/F | NO | NO |
| 186. | 112 | 32Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 188. | 114 | 90Y/F | NO | NO |
| 189. | 115 | 43Y/M | NO | NO |
| 190. | 116 | 54Y/M | NO | NO |
| 191. | 117 | 37Y/M | NO | NO |
| 192. | 118 | 69Y/M | NO | NO |
| 193. | 119 | 86Y/M | NO | NO |
| 194. | 120 | 10Y/F | NO | NO |
| 195. | 121 | 15Y/M | NO | NO |
| 196. | 122 | 70Y/M | NO | NO |
| 197. | 123 | 75Y/F | NO | NO |
| 198. | 157 | 38Y/F | NO | NO |

| | | | | |
|---|---|---|---|---|
| 199. | 158 | 78Y/M | NO | NO |
| 200. | 161 | 84Y/M | NO | NO |
| 201. | 165 | 48Y/M | YES | NO |
| 202. | 166 | 52Y/M | NO | NO |
| 203. | 167 | 05Y/F | NO | NO |
| 204. | 168 | 23Y/F | NO | NO |
| 205. | 169 | 24Y/F | NO | NO |
| 206. | 170 | 62Y/F | NO | NO |
| 207. | 171 | 65Y/F | NO | NO |
| 208. | 172 | 05Y/M | NO | NO |
| 209. | 173 | 24Y/M | NO | NO |
| 210. | 174 | 03Y/F | NO | NO |
| 211. | 175 | 32Y/M | NO | NO |
| 212. | 176 | 58Y/F | NO | NO |
| 213. | 177 | 86Y/M | NO | NO |
| 214. | 178 | 59Y/F | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 216. | 180 | 02Y/F | NO | NO |
| 217. | 181 | 38Y/M | NO | NO |
| 218. | 182 | 50Y/M | NO | NO |
| 219. | 183 | 40Y/M | NO | NO |
| 220. | 184 | 75Y/M | NO | NO |
| 221. | 185 | 44Y/M | NO | NO |
| 222. | 186 | 47Y/M | NO | NO |
| 223. | 187 | 37Y/M | NO | NO |
| 224. | 188 | 71Y/M | NO | NO |
| 225. | 189 | 52Y/F | YES | NO |
| 226. | 190 | 62Y/M | YES | NO |
| 227. | 191 | 84Y/F | NO | NO |
| 228. | 192 | 67Y/M | NO | NO |
| 229. | 194 | 56Y/F | NO | NO |
| 230. | 195 | 24Y/F | NO | NO |
| 231. | 196 | 61Y/M | YES | NO |
| 232. | 197 | 17Y/F | YES | NO |
| 233. | 198 | 19Y/M | NO | NO |
| 234. | 199 | 38Y/M | YES | NO |
| 235. | 200 | 43Y/F | YES | NO |
| 236. | 201 | 85Y/M | NO | NO |
| 237. | 202 | 50Y/M | YES | YES |
| 238. | 203 | 01Y/F | YES | NO |
| 239. | 204 | 41Y/M | NO | NO |
| 240. | 205 | 27Y/M | NO | NO |
| 241. | 206 | 87Y/F | YES | NO |
| 242. | 207 | 72Y/F | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 244. | 209 | 34Y/M | NO | NO |
| 245. | 210 | 37Y/F | YES | NO |
| 246. | 211 | 36Y/M | YES | YES |
| 247. | 212 | 70Y/F | NO | NO |
| 248. | 213 | 53Y/M | NO | NO |
| 249. | 214 | 80Y/M | NO | NO |
| 250. | 215 | 79Y/F | NO | NO |
| 251. | 216 | 06Y/M | NO | NO |
| 252. | 217 | 50Y/F | NO | NO |
| 253. | 218 | 30Y/M | NO | NO |
| 254. | 219 | 53Y/M | NO | NO |
| 255. | 220 | 49Y/M | NO | NO |
| 256. | 221 | 23Y/M | NO | NO |
| 257. | 222 | 64Y/M | YES | NO |
| 258. | 223 | 29Y/F | NO | NO |
| 259. | 224 | 60Y/F | NO | NO |
| 260. | 225 | 51Y/F | YES | NO |
| 261. | 226 | 35Y/M | NO | NO |
| 262. | 227 | 73Y/M | NO | NO |
| 263. | 228 | 38Y/M | NO | NO |
| 264. | 229 | 28Y/F | NO | NO |
| 265. | 230 | 50Y/F | NO | NO |
| 266. | 231 | 56Y/F | NO | NO |
| 267. | 232 | 50Y/F | NO | NO |

| | | | | |
|---|---|---|---|---|
| 268. | 233 | 75Y/M | NO | NO |
| 269. | 234 | 58Y/F | NO | NO |
| 270. | 235 | 80Y/F | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 272. | 237 | 43Y/M | NO | NO |
| 273. | 238 | 74Y/M | NO | NO |
| 274. | 239 | 71Y/M | NO | NO |
| 275. | 240 | 80Y/F | YES | NO |
| 276. | 241 | 25Y/M | NO | NO |
| 277. | 242 | 63Y/M | NO | NO |
| 278. | 243 | 48Y/M | NO | NO |
| 279. | 244 | 55Y/M | NO | NO |
| 280. | 245 | 29Y/F | NO | NO |
| 281. | 246 | 70Y/F | NO | NO |
| 282. | 247 | 63Y/M | NO | NO |
| 283. | 248 | 48Y/M | NO | NO |
| 284. | 249 | 11Y/F | NO | NO |
| 285. | 250 | 59Y/M | NO | NO |
| 286. | 251 | 63Y/F | NO | NO |
| 287. | 252 | 39Y/F | NO | NO |
| 288. | 253 | 27Y/F | NO | NO |
| 289. | 254 | 63Y/M | NO | NO |
| 290. | 255 | 66Y/M | NO | NO |
| 291. | 256 | 29Y/M | NO | NO |
| 292. | 258 | 42Y/M | NO | NO |
| 293. | 259 | 54Y/M | NO | NO |
| 294. | 260 | 15Y/F | NO | NO |
| 295. | 261 | 48Y/M | NO | NO |
| 296. | 262 | 39Y/M | NO | NO |
| 297. | 263 | 39Y/M | NO | NO |
| 298. | 264 | 30Y/M | NO | NO |
| **S.No.** | **Patient I.D.** | **Age/Sex** | **Inf A** | **H1N1** |
| 300. | 266 | 40Y/M | NO | NO |
| 301. | 267 | 30Y/M | NO | NO |
| 302. | 268 | 91Y/M | NO | NO |
| 303. | 269 | 73Y/M | NO | NO |
| 304. | 270 | 27Y/F | NO | NO |
| 305. | 271 | 32Y/M | NO | NO |
| 306. | 2072 | 48Y/M | NO | NO |
| 307. | 2073 | 53Y/M | NO | NO |
| 308. | 2074 | 60Y/F | NO | NO |
| 309. | 2022 | 30Y/F | YES | NO |
| 310. | 2018 | 73Y/F | YES | NO |
| 311. | 2000 | 33Y/M | YES | NO |
| 312. | 1972 | 56Y/M | YES | NO |
| 313. | 1902 | 55Y/M | YES | YES |
| 314. | 1901 | 52Y/M | YES | NO |
| 315. | 1904 | 48Y/F | YES | NO |
| 316. | 3312 | 04Y/M | YES | NO |
| 317. | 3315 | 18Y/F | YES | NO |
| 318. | 3318 | 37Y/F | YES | NO |
| 319. | 3319 | 51Y/M | YES | NO |
| 320. | 1857 | 02Y/M | YES | YES |
| 321. | 1845 | 59Y/M | YES | NO |
| 322. | 676 | 48Y/F | YES | YES |
| 323. | 677 | 36Y/M | YES | YES |
| 324. | 655 | 32Y/F | YES | YES |

## VI. DATA ANALYSIS

**Table : No.** and percentage of positive and negative H1N1 Samples

| **Result** | **No. of Samples (N=324)** | **Percentage** |
|---|---|---|
| Positive | 8 | 2.46% |
| Negative | 316 | 97.53% |

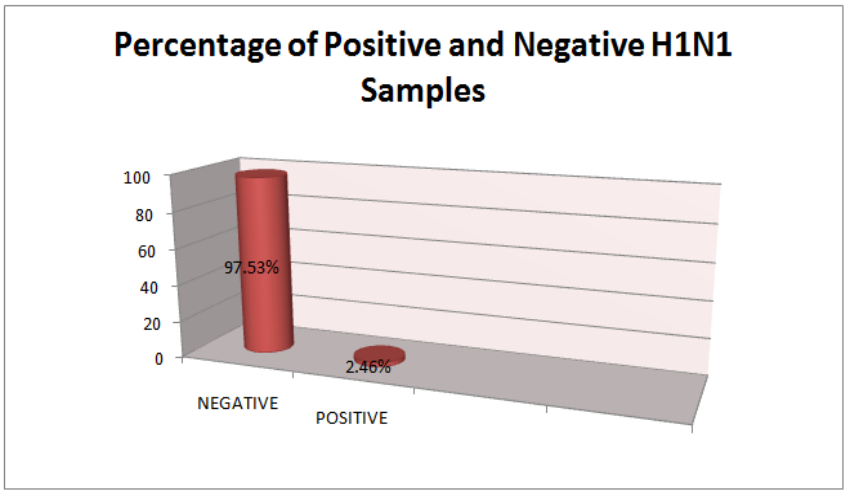



**Table: No**. and Percentage of samples positive for Influenza A but negative for H1N1

| Result | No. of Samples N=324 | Positive Rate (%) |
|---|---|---|
| Positive | 31 | 9.57% |
| Negative | 293 | 90.43% |

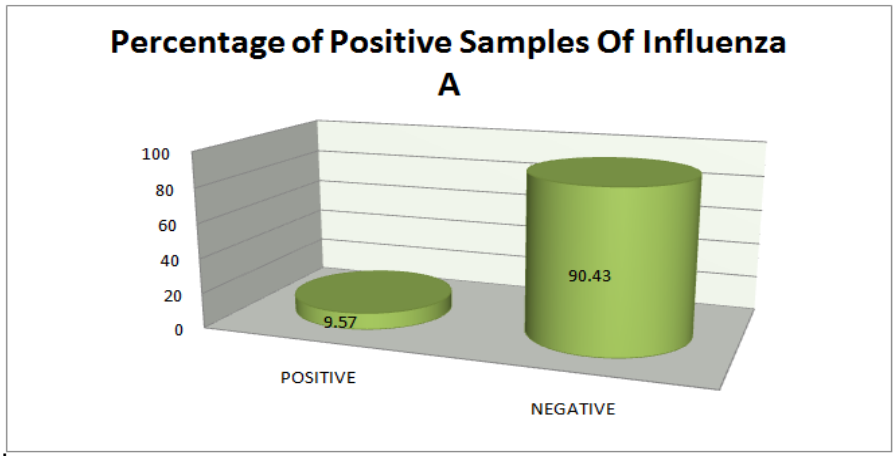



**Table :** Positivity rate of H1N1 virus in Males and Females

| Gender | Total no. of Samples | Positive Samples | Positivity Rate (%) |
|---|---|---|---|
| Male | 176 | 5 | 2.84% |
| Female | 148 | 3 | 2.03% |

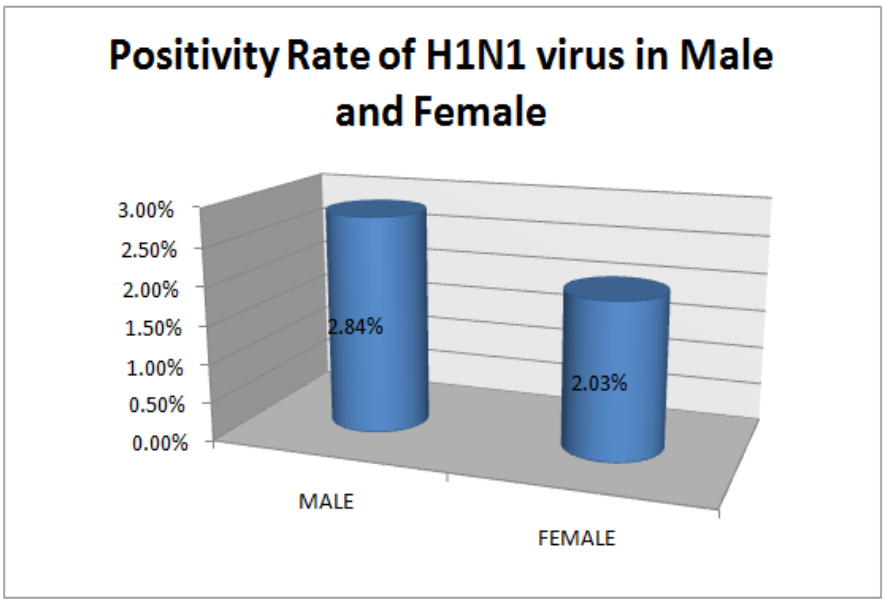
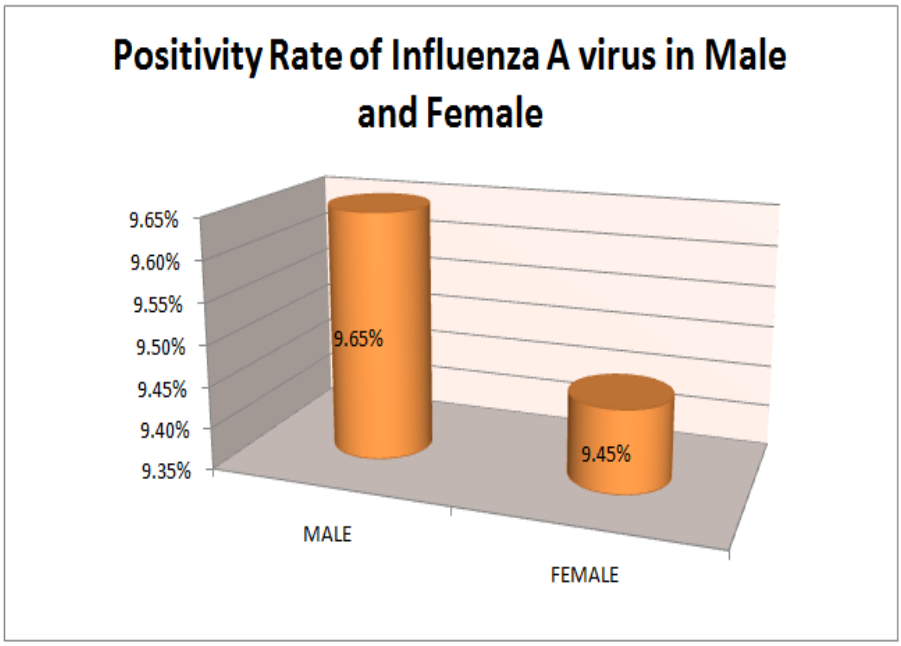



**Table 5:** Positivity Rate of Influenza A in Male and Female

| Gender | Total no. of samples | No. of positive samples | Positivity Rate (%) |
|---|---|---|---|
| MALE | 176 | 17 | 9.65% |
| FEMALE | 148 | 14 | 9.45% |




## VII. Conclusion

We have collected 324 samples from different collection centers in Delhi and Jaipur.
Further isolating the RNA with Qiagen kit and detecting the specific targets for H1N1 virus we can conclude our work with the following points.

- Only 2.46% of samples showed Positive results for H1N1 while other
- 97.53% were negative during 2011-2012.
- 9.57% of the patients were found infected by Influenza A only.
- Positivity rate was found maximum in Males 2.84% in comparison with Females 2.03% in case of H1N1.
- Positivity rate was found maximum in Male 9.65% as compared to Female 9.45% in case of Influenza A.

## DECLARATION

I do hereby declare that the thesis entitled " **To DETECTION OF H1N1 (SWINE FLU) VIRUS BY REAL TIME PCR TECHNOLOGY "** submitted *to* 'Biotech Consortium India Limited' in partial fulfillment of the "Biotech Industrial Training Programme" is a faithful record of project work carried by Anil kumar, under the guidance and supervision of Dr. Yogesh Kumar Singh and Mr. Mukesh Kumar.

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## REFERENCES

(1) www.infocusrx.com

(2) Reid AH, Taubenberger JK. The origin of the 1918 pandemic influenza virus: a continuing enigma. J Gen Virol 2003; 84: 2285–2292

(3) Lamb R, Krug R. Orthomyxoviridae: The viruses and their replication. In: Knipe D, Howley P, eds: Field's Virology. 4th Ed. Philadelphia: Lippincott, Williams and Wilkins; pp. 1487–1531

(4) Reid AH, Taubenberger JK. The origin of the 1918 pandemic influenza virus: a continuing enigma. J Gen Virol 2003; 84: 2285–2292

(5) Ludwig S, Stitz L, Planz O, Van H, Fitch W, Scholtissek C. European swine virus as a possible source for the next influenza pandemic? Virology 1995; 212: 555–561.

(6) Reid AH, Taubenberger JK. The origin of the 1918 pandemic influenza virus: a continuing enigma. J Gen Virol 2003; 84: 2285–2292

(7) www,scribd.com

(8) www.virology.us

(9) 2009 H1N1 flu: international situation update, 22 January 2010; http://www.cdc.gov/h1n1flu/updates/international

(10) Jameel, S., The 2009 influenza pandemic. *Curr. Sci.*, 2010, 98, 306–311

(11) World Health Organization, Pandemic (H1N1) 2009 – update 96, 16 April 2010; http://www.who.int/csr/don/2010_04_16/en/index.html.

(12) Ministry of Health and Family Welfare, Government of India, Situation update on H1N1, 11 April 2010; http://mohfwh1n1.nic.in/documents/PDF/SituationalUpdatesArchives/april2010/Situational%20Updates%20on%2011.04.2010.pdf

(13) Boëlle, P. Y., Bernillon, P. and Desenclos, J. C., A preliminary estimation of the reproduction ratio for new influenza A(H1N1) from the outbreak in Mexico, March–April 2009. *Euro Surveill.*, 2009, 14(19), pii: 19205

(14) Cruz-Pacheco, G. *et al.*, Modelling of the influenza A(H1N1)v outbreak in Mexico City, April–May 2009, with control sanitary measures. *Euro Surveill.*, 2009, 14(26), pii: 19254

(15) Fraser, C. *et al.*, WHO Rapid Pandemic Assessment Collaboration, Pandemic potential of a strain of influenza A(H1N1): early findings. *Science*, 2009, 324, 1557–1561

(16) Yang, Y. *et al.*, The transmissibility and control of pandemic influenza A(H1N1) virus. *Science*, 2009, 326, 729–733

(17) McBryde, E. *et al.*, Early transmission characteristics of influenza A(H1N1)v in Australia: Victorian state, 16 May–3 June 2009. *Euro Surveill.*, 2009, 14(42), pii: 19363.

(18) Trifonov, V., Khiabanian, H., & Rabadan, R. (2009). Geographic Dependence, Surveillance, and Origins of the 2009 Influenza A (H1N1) Virus *New England Journal of Medicine* DOI: 10.1056/NEJMp0904572

(19) ^ Smith AE, Helenius A (April 2004). "How viruses enter animal cells". Science 304 (5668): 237)

(20) Bouvier NM, Palese P (September 2008).

(21)Vaccine 26,Suppl4:D4953. doi:10.1016/j.vaccine.2008.07.039. PMID 192301603

(22) Wagner, R; Matrosovich M, Klenk H (May–June 2002).

(23) *Rev Med Virol* **12** (3): 159–66.doi:10.1002/rmv.352. PMID 11987141

(24) Steinhauer DA (May 1999). "Role of hemagglutinin cleavage for the pathogenicity of influenza virus". *Virology* **258** (1): 1–20. doi:10.1006/viro.1999.9716. PMID 10329563.

(25) Bouvier NM, Palese P (September 2008). "The biology of influenza viruses". *Vaccine* **26** **Suppl** **4**: D49–53. doi:10.1016/j.vaccine.2008.07.039. PMID 19230160.

(26) Pinto LH, Lamb RA (April 2006). "The M2 proton channels of influenza A and B viruses". *J. Biol. Chem.* **281** (14): 8997–9000. doi:10.1074/jbc.R500020200. PMID 16407184

(27) Cros, J; Palese P (September 2003). "Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto and Borna disease viruses". *Virus Res* **95** (1–2): 3–12.doi:10.1016/S0168-1702(03)00159-X. PMID 12921991

(28) Kash, J; Goodman A, Korth M, Katze M (July 2006). "Hijacking of the host-cell response and translational control during influenza virus infection". *Virus Res* **119** (1): 111–20.doi:10.1016/j.virusres.2005.10.013. PMID 16630668.

(29) Nayak, D; Hui E, Barman S (December 2004). "Assembly and budding of influenza virus". *Virus Res* **106** (2): 147–65. doi:10.1016/j.virusres.2004.08.012. PMID 15567494.

(30) Wagner, R; Matrosovich M, Klenk H (May–June 2002). "Functional balance between haemagglutinin and neuraminidase in influenza virus infections". *Rev Med Virol* **12** (3): 159–66.doi:10.1002/rmv.352. PMID 11987141

(31)Wilson, J; von Itzstein M (July 2003). "Recent strategies in the search for new anti-influenza therapies". Curr Drug Targets 4 (5): 389–408. doi:10.2174/1389450033491019.PMID 12816348.

(32) Drake, J (1 May 1993). "Rates of spontaneous mutation among RNA viruses". *Proc Natl Acad Sci USA* **90** (9): 4171

(33) Hilleman, M (August 19 2002). "Realities and enigmas of human viral influenza: pathogenesis, epidemiology and control". *Vaccine* **20** (25–26): 3068–87. doi:10.1016/S0264-410X(02)00254-2. PMID 12163258

(34) Call S, Vollenweider M, Hornung C, Simel D, McKinney W (2005). "Does this patient have influenza?". *JAMA* **293** (8): 987–97. doi:10.1001/jama.293.8.987. PMID 15728170.

(35) Suzuki E, Ichihara K, Johnson AM (January 2007). "Natural course of fever during influenza virus infection in children". *Clin Pediatr (Phila)* **46** (1): 76–9. doi:10.1177/0009922806289588.PMID 17164515.

(36) "Influenza: Viral Infections: Merck Manual Home Edition". www.merck.com. Retrieved 2008-03-15.

(37) Carrat F, Luong J, Lao H, Sallé A, Lajaunie C, Wackernagel H (2006). "A 'small-world-like' model for comparing interventions aimed at preventing and controlling influenza pandemics".*BMC Med* **4**: 26. doi:10.1186/1741-7015-4-26. PMID 17059593

(38) "CDC H1N1 Flu : Updated Interim Recommendations for the Use of Antiviral Medications in the Treatment and Prevention of Influenza for the 2009-2010 Season". Centers for Disease Control and Prevention.

(39) Mitamura K, Sugaya N (2006). "[Diagnosis and Treatment of influenza—clinical investigation onviral shedding in children with influenza]". *Uirusu* **56** (1): 109–16. doi:10.2222/jsv.56.109.PMID 17038819.

(40) Grassly NC, Fraser C (June 2008). "Mathematical models of infectious disease transmission".*Nat.Rev.Microbiol.* **6** (6):47787. doi:10.1038/nrmicro1845. PMID 1853 3288.

(41) Weber TP, Stilianakis NI (November 2008). "Inactivation of influenza A viruses in the environment and modes of transmission: a critical review". *J. Infect.* **57** (5): 361–73.doi:10.1016/j.jinf.2008.08.013. PMID 18848358.

(42) Hall CB (August 2007). "The spread of influenza and other respiratory viruses: complexities and conjectures". *Clin. Infect. Dis.* **45** (3): 353–9. doi:10.1086/519433. PMID 17599315

(43) Brankston G, Gitterman L, Hirji Z, Lemieux C, Gardam M (April 2007). "Transmission of influenza A in human beings". *Lancet Infect Dis* **7** (4): 257–65. doi:10.1016/S1473-3099(07)70029-4. PMID 17376383.

(44) Tellier R (November 2006). "Review of aerosol transmission of influenza A virus". *Emerging Infect. Dis.* **12** (11): 1657–62. PMID 17283614.

(45) Cole E, Cook C (1998). "Characterization of infectious aerosols in health care facilities: an aid to effective engineering controls and preventive strategies". *Am J Infect Control* **26** (4): 453–64.doi:10.1016/S0196-6553(98)70046-X. PMID 9721404.

(46) Weber TP, Stilianakis NI (November 2008). "Inactivation of influenza A viruses in the environment and modes of transmission: a critical review". *J. Infect.* **57** (5): 361–73.doi:10.1016/j.jinf.2008.08.013. PMID 18848358.

(47) Thomas Y, Vogel G, Wunderli W, *et al.* (May 2008). "Survival of influenza virus on banknotes". *Appl. Environ. Microbiol.* **74** (10): 3002–7. doi:10.1128/AEM.00076-08.PMID 18359825.

(48) "Influenza: Viral Infections: Merck Manual Home Edition". www.merck.com. Retrieved 2008-03-15.

(49) Bean B, Moore BM, Sterner B, Peterson LR, Gerding DN, Balfour HH (July 1982). "Survival of influenza viruses on environmental surfaces". *J. Infect. Dis.* **146** (1): 47–51. PMID 6282993.

(50) Weber TP, Stilianakis NI (November 2008). "Inactivation of influenza A viruses in the environment and modes of transmission: a critical review". *J. Infect.* **57** (5): 361–73.doi:10.1016/j.jinf.2008.08.013. PMID 18848358 & ref:(47)

(51) "Influenza Factsheet". Center for Food Security and Public Health, Iowa State University. p. 7

(52) (Ref: www.flu.gov.com)

(53) (Ref: www.pnas.org)

(54) (Ref – www.Flu.Gov.com)

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