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REVIEW ARTICLE

Zoonosis Update on H9N2 Avian Influenza Virus

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ABSTRACT

Influenza A viruses infect various mammals like human, horse, pig and birds as well. A total of 16 hemagglutinin (HA) and 9 neuraminidase (NA) subtypes have been identified. Most of the combinations are found in birds and relatively few have been isolated from mammals. Although there is no report of human to human transmission till to date, several cases of H5N1, H7N7 and H9N2 identified in humans since 1997 raised serious concern for health and veterinary profession. This review paper will focus H9N2 avian influenza virus (AIV) with special emphasis on zoonosis. The virus H9N2 though not highly pathogenic like H5N1 but can be virulent through antigenic drift and shift.

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INTRODUCTION

Avian influenza (AI) viruses are typed as high pathogenic avian influenza (HPAI) or low pathogenic avian influenza. Office des International Epizooties (OIE) set up molecular criteria to identify HPAI. This is based on amino acid sequence on HA cleavage site. *In vivo* it will be considered as HPAI if any isolate can kill at least 75% of susceptible 4-6 weeks old chicken within 10 days post inoculation by i/v route (OIE, 2012). AI have the tendency to change its genetic makeup and able to cause interspecies transmission with zoonotic potential (Aamir *et al*., 2007; Cheema *et al*., 2011; Khan *et al*., 2011; Siddique *et al*., 2012). It can be occurred by two ways: random mutations in the genome especially in hemagglutinin or by reassortment of gene segments (Lipsitch *et al*., 2012). Based on serological and experimental studies in rodents (especially mice and hamsters), carnivores, ungulate ruminants can be infected with influenza A virus (Swayne and King, 2003). Transmission of AIV to human being may occur without its binding preferences for α 2,6 linkage due to shared non glycoprotein gene constellation which cause limited human infection in Hong Kong and China since 1997 (Blair *et al*., 2013). Like Influenza A virus many of the zoonotic viral disease which has ability to transmit between human and animal possess RNA genomes (Holmes, 2009). With a low fidelity of having lack of exonuclease proofreading ability Influenza A virus (IAV) exists as a quasi-species (Furuse *et al*., 2010). IAVs can adopt stably in a variety of animal including human and birds. Novel human adapted AIV can cause pandemic since 100 years (Taubenberger and Morens, 2009). AIV do not replicate well in mammals indicating birds to human transmission is unlikely to occur. However, since 1997 several cases of H5N1, H7N7 and H9N2 identified in human raised concern in health and veterinary professions. In this paper we reviewed current knowledge of AIV H9N2 subtype with special emphasis on zoonosis.

Morphology: Avian influenza virus belongs to the family Orthomyxoviridae (Palese and Shaw, 2007). Shape of Avian Influenza virus (AIV) varies from spherical to filamentous and diameter ranges from 80-120 nm. From the viral envelope HA & NA are projected (Palese and Shaw, 2007). Till to date 16 HA & 9 NA subtypes have been identified. Theoretically 144 possible combinations of HA-NA subtype are possible. At least 116 of subtypes have been isolated from birds (Krauss *et al*., 2004; Munster *et al*., 2007). Size of HA is 10-14 nm and NA 4-6 nm in diameter. The virus belongs to helical symmetry. It contains ssRNA with negative sense. Its genome is segmented. It has eight segments. At least 11 ORF (open reading frame) are coded by 8 segments. Its genome codes for 10 proteins. Out of 10, eight help in structure of virus (HA, NA, NP, M1, M2, PB1, PB2 and HA), and rest two

are nonstructural proteins (NS1 and NS2) which express only during replication of virus in the host cytoplasm (Palese and Shaw, 2007). Most ample matrix protein M1 is settled beneath the viral envelope. Virus makes the envelope by taking materials from the infected cell membrane and contains multiple copies of the HA and NA glycoproteins (Swayne and Halvorson, 2008). Viral envelope is chiefly made by cholesterol and glycosphingolipid. It is derived from the host cell membrane (Swayne *et al*., 2008).

HA, NA and M2 (ion channel) protein are hooked in the lipid layer of envelope. HA is grouped into type I transmembrane protein and it constitutes about 80% of the envelope protein. HA is glycosylated and acts as fusion and receptor protein. AIV adapted to birds have α 2-3 sialic acid receptors while in human it has specificity α 2-6 receptors. After binding with the receptor virus is internalized into acidic compartment leads to conformational change of HA. In birds it is cleaved by exogenous serine protease and in mammals by tryptase which is produced by clara cells of bronchiolar epithelium. HA mediates in the attachment of virus with the susceptible cell and provokes neutralizing antibodies. Fusion and virus infectivity depends on cleavability of HA. NA is grouped into type II transmembrane protein. Its shape is homotetramer which is hooked into viral envelope. NA helps in the elution of virus from the host cell and spread of virus through host. M2 is grouped into III category of transmembrane protein which acts as an ion channel (Swayne and Halvorson, 2008). M2 is required for viral replication and antiviral drug adamantine acts on it. Segmented RNA of AIV is wrapped by nucleo protein (NP). There are 3 polymerase proteins present in the AIV. They are designated as PB1, PB2 and PA respectively. PB1 serves as RNA dependent RNA polymerase, mRNA is synthesized by PB2. PA is required for endonucleolytic cleavage of host RNAs (Jeffery and John, 2010). Lipid content of the viral envelope plays an important role in entry of virus into a susceptible cell. Role of cholesterol in viral envelope was assessed by using methyl β cyclodextrin depletion assay. It reduces cholesterol from the envelope of virus and reduces virus infection considerably. While on naked Simian virus 40 did not show any effect. Moreover the envelope cholesterol reduction remarkably affects the process of influenza virus fusion which is measured by fluorescence dequenching assays (Sun and Whittaker, 2003).

The influenza virus contains a segmented negative sense RNA genome. It buds from the apical portion of the epithelium. Mature virion is not formed within cell. Virus comprises 3 major components which are envelope, matrix and core. The viral envelope encompasses viral ribonucleo protein (RNP) consists of lipid bilayer which is embedded by spikes build by glycol proteins. Embedded M1 protein makes bridge between viral core and envelope. Viral core is made up by RNP, RNA, NP (nucleoprotein), NEP (nuclear export protein) and polymerase complex (PA, PB1 and PB2). Transmembrane viral proteins are available in the assembly place of cell membrane through exocytic pathway. HA and NA both use lipid as a vehicle across the cell surface. These lipids which act as vehicle are chiefly composed of cholesterol and glycosphingo lipid. Viral nucleic acids are manufactured in the nucleus of cell and exported into the cytoplasm through nuclear pore (Nayak *et al*., 2004).

Matrix is made up of two proteins, M1 and M2. M2 protein mediates cell penetration, ion channel activity and uncoating of nucleic acid (Swayne *et al*., 2008). Although in another study it shows virus lacking M2 can replicate without compromising its infectivity (Watanabe *et al*., 2001). Nucleo protein serves as structural protein in ribo nucleo protein (RNPs). It helps in transcription and trafficking of RNP between cytoplasm and nucleus. NS1 has multiple functions such as increase viral mRNA translocation, type I IFN antagonist, inhibiting host mRNA processing (Palese and Shaw, 2007). NS2 (nuclear export protein) facilitates export of viral RNP.

Molecular Characterization: In laboratory AIV is diagnosed by isolating the virus in SPF (specific pathogen free) embryonated eggs followed by HI test and NA assay on harvested allantoic fluid (OIE, 2012). A variety of PCR can be used to diagnose different variants of AIV based on matrix or nucleoprotein gene (Kiss *et al*., 2006). Pandemic preparedness is carried in the world wide after detection of any novel subtype of HA. Gene analysis of 2 human H9N2 isolates from Hong Kong revealed that it closely resembles with an H9N2 isolate from quail. It also contains the genes of isolate of H5N1 virus (Saito *et al*., 2001). The human isolate produced respiratory infection in Syrian hamster suggesting that it can replicate in mammalian host. The 12 genes of NA, H9N2 influenza virus strains were isolated from infected poultry in several farms in mainland China during 1995–2002 were amplified and sequenced. It showed that neuraminidase in 12 strains had a deletion of 3 amino acid residues at positions 63–65 as compared to that of A/turkey/Wisconsin/189/66, however the isolates H9N2 of Korea and Pakistan had no deletion. Phylogenetic analysis exhibited that these isolates NA gene were related to a duck isolate. The viruses which have been isolated show NA gene of the H9N2 viruses belonged to different lineage from those of the 12 isolates (Jin *et al*., 2006). The results displayed that the gene sequence of NA in H9N2 strains which have been isolated in China for the last 8 years well conserved. HI assay revealed that 18 out of 20 isolates showed cross reactivity with antisera raised against 3 isolate. However antigenic diversity among the isolates was observed. The antisera did not show cross reactivity against two other isolates. By phylogenetic analysis it was recorded that 19 out of 20 isolates were related to Y-280 sublineage of Eurasia (Chengjun *et al*., 2005). One isolate is closely associated with a turkey isolate which belongs to North American Lineage.

Seven H9N2 viruses were isolated in the UAE (United Arab Emirates) during 2000-2003. Phylogenetic relation of these viruses was studied by comparing with the isolated virus in the Middle East and other Asian countries. An additional basic amino acid is found at the cleavage site of all the viruses. Six out of seven isolates have mutation and increased tendency toward human receptors (Aamir *et al*., 2007). More than 90% similarity was observed in surface glycoprotein and most internal gene of virus were similar to quail isolate. Four amino acid substitutions were observed in the hemadsorbing site

of neuraminidase. During 2003-2004, 29 H9N2 AIV isolates were obtained from different states of India. Subtyping of the virus was carried out by neuraminidase inhibition assay, HI and RT-PCR. On sequencing of HA gene it showed that isolates were very closely related (95-99.6%). The isolates showed 92-96% homology with other isolates from Asia and Germany. On amino acid sequences it shows that it has a tendency to bind with α 2-6 sialic acid receptor (Nagarajan *et al*., 2009). Four out of six isolates showed glycyosylation at 5th position in HA1 cleavability. But remaining two showed at 7th position of glycosylation site. On phylogenetic analysis it showed that it had a resemblance with quail isolate. Twelve H9N2 viruses were isolated in Pakistan in the year 2005-2009. The HA sequences showed that it contain leucine instead of glutamine at position 226 which is a recognized indicator for α 2-6 galactose receptor (Iqbal *et al*., 2009). The NS gene showed considerable genetic variation. It showed analogy to H5 or H7 subtype rather than H9N2 subtype.

Immune response to AIV: AIV elicits host immune response resulting abortion of virus replication. In period of 21st century in 2009, new influenza A/ H1N1 virus of swine origin causing first pandemic in the world (WHO, 2009). Besides H5N1, H7N7, and H9N2 of avian origin sporadically transmitted from animal to human being (de Wit *et al*., 2008).

AIV infection is first confronted by innate immune system. Initially viral RNA is recognized by pattern recognition receptors. It includes retinoic acid inducible gene -1 (RIG-1) (Pang and Iwasaki, 2011). Viral RNA binds with toll like receptors (TLR7) which provoke production of cytokines and type I IFN (Lund *et al*., 2004). IFN-α, IFN-ß have strong antiviral activity. Viral replication and protein synthesis are blocked by this interferon (Sato *et al*., 1998).

Role of alveolar macrophage in influenza virus infection: Soon after infection, alveolar macrophage activate and phagocytose (apoptotic) influenza virus infected cell and confine virus disseminate (Tumpey *et al*., 2005; Kim *et al*., 2008). Once macrophage activated it produces TNF α, nitric oxide synthase 2 (NOS2) and leads to influenza virus mediated damage (Jayasekera *et al*., 2006; Lin *et al*., 2008). Blood derived macrophage infected with influenza H5N1 produce more cytokines in contrast to alveolar macrophages (van Riel *et al*., 2011; Khan *et al*., 2011).

Dendritic cells and their role in influenza specific immunity: In influenza virus infections dendritic cells (DC) are regarded as professional antigen presenting cells. The conventional DCs (cDC) are located beneath respiratory epithelium. It can detect and opsonize virion during infection. Upon entry of AIV conventional DCs present antigen to T cells to activate them (GeurtsvanKessel and Lambrecht, 2008; Hintzen *et al*., 2006). Viral proteins are degraded by dendritic cells and subsequently MHC class I or class II molecules present the epitopes. Viral epitopes are degraded by proteasome and liberated into cytosol. Subsequently it is transported to endoplasmic reticulum along with MHC class molecules. For MHC II presentations viral antigens are degraded by lysosome in endosome ((CD11+ DC) (GeurtsvanKessel *et al*., 2009 a,b).

Adaptive Immune System: Influenza virus elicits virus specific antibody (Mancini *et al*., 2011). Surface viral proteins HA and NA are important as these produce protective immunity (Gerhard, 2001). Antibodies against HA prevent the attachment of virus into host cell. It can neutralize the virus. It also facilitates phagocytosis by expressing Fc receptors in the cell (de Jong *et al*., 2000). Antibodies against NA have a potential protective role against AIV infection. Antibodies bind with NA inhibit the spread of virus to the adjacent cell. It also facilitates antibody dependent cell mediated cytotoxicity (Mozdzawoska *et al*., 1999). Antibodies against nucleoprotein (NP) may contribute to protect against avian influenza infection (Carragher *et al*., 2008). Exact role of antibodies is yet to be elucidated. It is believed that it can provoke complement associated lysis of infected cell (Sambhara *et al*., 2001).

Cellular immunity: CD4, CD8 and T regulatory cells are activated by AIV infection. APCs associated with class II MHC molecules present viral peptides to CD4 cells. Infected cells are lysed by some CD4 cells (Soghoian and Streeck, 2010). IL-4, IL-13 are produced by Th2 cells which promotes B cell response (Lamb *et al*., 1982; Wright *et al*., 2007). IFN-γ, IL-2 are produced by Th1 cells which leads to cellular immune response. Influenza virus infections activate CD8+ cells and more to site of infection. Influenza virus infected cells are recognized there by CD8 cells and inhibit the production of progeny virion. Perforin and granzyme mediate the lysis of infected cells. Permeability of infected cells is increased by perforin and subsequently granzyme leads to apoptosis of the cell (Metkar *et al*., 2008; Regner *et al*., 2009).

H9N2 AIV infections in Human Beings: H9N2 is one of the major subtype of avian influenza virus which is prevailing among domestic poultry since 1990s (Xu *et al*., 2007). Three human infections of H9N2 AIV have been reported in Hong Kong between 1999 and 2003 (Butt *et al*., 2005). In a sero survey from 150 blood donors in Hong Kong, three individuals had neutralizing antibodies against H9N2 (Peiris *et al*., 1999). The capacity of H9 HA surface glycoprotein to bind with the avian and human being receptors highlighting it may lead to influenza pandemic (Peiris *et al*., 2001). Analysis of 75 human serum samples in USA revealed that 93.3%, 5.3% and 1.3% individuals had <1:10, 1:10 and 1:20 antibody titer against H9 avian influenza virus (Kayali *et al*., 2008). In China, highest antibodies against H9 were reported in poultry retailers (15.9%) as compared to other worker groups associated with poultry industry (Wang *et al*., 2009). In Xinjiang and Liaoning provinces of China, 1.7 and 1%, respectively seropositivity against H9 virus was recorded when sera of poultry workers were analyzed through HI test using horse RBCs (Jia *et al*., 2009). A sero survey conducted on (n=300) exposed individuals with respiratory signs and healthy unexposed individuals in Fars province of Iran revealed higher prevalence of H9N2 in exposed individuals i.e. butchers and veterinarians

(Hadipour, 2010; Hadipour *et al*., 2011). A seroprevalence of 85.7% and 30.4% against H9 was recorded for vaccinators and veterinarians, respectively in Punjab province of Pakistan. The higher prevalence rate in vaccinators may be attributed to their longer duration of exposure as compared to veterinarians. By district wise the highest seroprevalence (82.1%) was recorded in Toba Tek Singh whereas the lowest (9.7%) was recorded in Islamabad (Ahad *et al*., 2013).

Intranasal infection of H9N2 in hamster produced mild syndrome. Virus was detected in trachea and lungs. Sero conversion was detected after 14 days and antibody persisted for at least 28 weeks (Qi *et al*., 2011). Occurrence of H9N2 virus infection in two immunocompromised individuals revealed that virus can also act as opportunistic agent (Cheng *et al*., 2011). Blair *et al*. (2013) reported significantly higher incidence of H9N2 in old age individuals (≥ 60 years) of Cambodia as compared to younger ones. The lower incidence in younger individuals may be attributed to stronger innate immunity in this age which can efficiently eliminate viral infection and subsequent production of specific antibodies. 12 isolates of H9N2 from Pakistan have been studied. It was revealed that nonstructural (NS) gene showed greater diversity and it closely resembles with H5 and H7 subtypes rather than established H9N2 Eurasian lineage. The novel gene possessed by H9N2 subtype posed a threat of human pandemics (Iqbal *et al*., 2009).

Conclusion: An estimated 75% emerging infectious diseases are zoonotic, mainly of virus origin. Wild life constitutes a large often unknown mysterious reservoir of infectious agents which can be source of reemergence of previously controlled zoonosis. The pathobiology of influenza virus is intrigue and complex. Its intricacy is multifaceted like genetic make up of the virus, interaction of virus with its host, its genetic diversity and evolution with the passage of time. Although we unraveled some of the basic questions of the virus but numerous challenges have not yet been solved. Future progress requires better understanding of structure and functional relation of all protein of the virus.

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