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Review

In vivo reassortment of influenza viruses

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The genetic material of influenza A virus consists of eight negative-sense RNA segments. Under suitable conditions, the segmented structure of the viral genome allows an exchange of the individual gene segments between different strains, causing formation of new reassorted viruses. For reassortment to occur, co-infection with two or more influenza virus strains is necessary. The reassortment is an important evolutionary mechanism which can result in antigenic shifts that modify host range, pathology, and transmission of the influenza A viruses. In this process, the influenza virus strain with epidemic and/or pandemic potential can be created. Cases of this kind were in 1957 (Asian flu), 1968 (Hong Kong flu) and recently in 2009 (Mexico). Viruses containing genes of avian, swine, and/or human origin are widespread around the world, for example the triple reassortant H1N1 virus causing the 2009 influenza pandemic in 2009 that has become a seasonal virus. The aim of the study is to present the mechanism of reassortment and the results of experimental co-infection with different influenza viruses.

Key words: influenza A viruses

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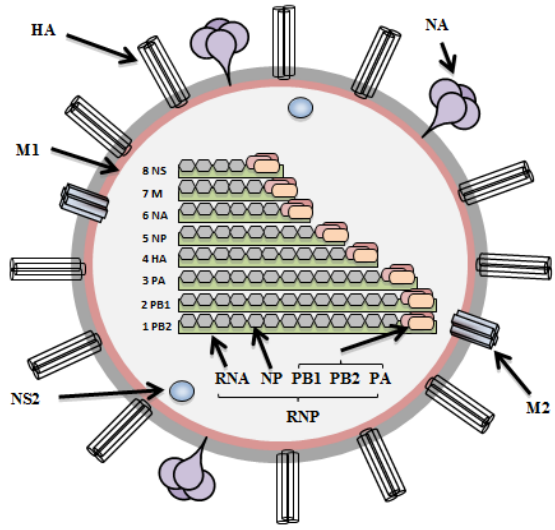

Figure 1. Diagrammatic presentation of an IAV particle.
Membrane proteins: haemagglutinin (HA), neuraminidase (NA) and small ion channel protein — matrix 2 (M2). Matrix 1 protein (M1) surrounding eight segments of single stranded RNA, individually combined with proteins (NP — nucleoprotein; PB1, PB2, PA — polymerase subunits) into ribonucleoprotein complexes (RNP). Non-structural protein 2 (NS2) is contained within the virion, whereas the non-structural protein 1 (NS1) is not.

INTRODUCTION

Influenza A viruses (IAV) which belong to the *Orthomyxoviridea* family, are classified into subtypes based on the two surface glycoproteins: haemagglutinin — HA and neuraminidase — NA. Currently, eighteen HA and eleven NA subtypes of IAV have been circulating in nature (Gerber *et al.*, 2014). Most of them have been isolated from water birds, whereas certain subtypes infect various avian and mammalian species. The emergence of novel IAVs results from accumulation of point mutations in their genetic material (genetic drift), as well as from the reassortment of their gene segments (genetic shift). It is believed that pig plays an essential role in the reassortment of IAVs due to its susceptibility to the majority IAV subtypes and also because of the presence in its respiratory tract receptors for both, avian and mammalian viruses (Taubenberger & Kash, 2010).

GENOME STRUCTURE

The structure of IAVs genome favours exchange of individual ribonucleic acid (RNA) molecules between IAVs. The genome of the IAV consists of eight single stranded *RNA chains* of negative polarity that together with nucleoprotein (NP) monomers and the virus polymerase form ribonucleoprotein complexes (RNPs) (Resa-Infante *et al.*, 2011; Lu *et al.*, 2014). Within the RNPs, RNA molecules are the genetic material of the virus, NP monomers cover and protect it, whereas the polymerase is responsible for the transcription and replication (Resa-Infante *et al.*, 2011; Tarus *et al.*, 2012). The IAV synthetize 10 essential viral proteins, as well as several additional ones (Gerber *et al.*, 2014) (Table 1). Each RNA segment encodes specific viral protein (Fig. 1) (Lu *et al.*, 2014). Among all RNA chains, two of them: segment 7 (the M gene) and 8 (the NS gene) are transcribed to messenger RNAs (mRNAs), which are spliced into two chains encoding M1 and M2, NS1 and NS2, respectively (Palese & Shaw, 2007). Additionally, most of IAVs have at the 5' end of second RNA segment an alter-

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Abbreviations: IAV, influenza A virus; RNA, ribonucleic acid; mRNA, messenger ribonucleic acid; cRNA, complementary ribonucleic acid; vRNA, viral ribonucleic acid; RNP, ribonucleoprotein; HA, haemagglutinin; NA, neuraminidase; MDCK cell line, Madin-Darby canine kidney cell line NP, nucleoprotein; M gene, matrix gene; M1, matrix protein 1; M2, matrix protein 2; NS1, non-structural protein 1; NS2, non-structural protein 2; PB1, basic protein 1; PB1-F2, basic protein 1-frame 2; PB2, basic protein 2; PA, acid protein

Table 1. RNA segments and their encoded proteins (Goto & Kawaoka, 2001; Palese & Shaw, 2007; Gerber *et al*., 2014).

Segment	Nucleotids	Protein
1	2341	basic protein 2 (PB2)
2	2341	basic protein 1 (PB1)
		PB1-F2*
		N40*
3	2233	acid protein (PA)
		PA-X*
		PA-N155*
		PA-N182*
4	1778	hemaglutinin (HA)
5	1565	nucleoprotein (NP)
6	1413	neuraminidase (NA)
7	1027	matrix protein 1 (M1)
		M2
		M43*
8	890	non-structural protein 1 (NS1)
		NS2
		NS3*

*Auxiliary proteins.

native open reading frame (the PB1 gene) that encodes the 87-amino acid long PB1-F2 polypeptide (Chen *et al*., 2001; Palese & Shaw, 2007) (Table 1).

VIRAL REPLICATION

To initiate replication cycle, the IAV needs to enter the host cell. The first step is the attachment of the IAV to the cell surface. The virus recognises and binds to the receptors (sialic acids) by its surface glycoprotein, the HA (Palese & Shaw, 2007; de Vries *et al*., 2011). After the attachment the following step is clathrin-mediated endocytosis (de Vries *et al*., 2011). However, Sieczkarski & Whittaker (2002) and de Vries *et al*. (2011) suggested the alternative model for the IAV cell entry: a non-clathrin, non-caveolae pathway and macropinocytosis route, respectively. The fusion of endosomal and IAV membranes is induced by a structural change of the cleaved HA activated by the low pH (~5), which results in RNPs release into the cytoplasm of the infected cell (Lakadamyali *et al*., 2004; Palese & Shaw, 2007). Then, the IAV genome, due to the nucleocytoplasmic transfer mechanism of the host cell, is transported to the nucleus (Palese & Shaw, 2007; Resa-Infante *et al*., 2011). The NP and all polymerase proteins possess nuclear localization signals required for the nuclear import of RNPs (Reperant *et al*., 2012). After entering the nucleus, the first step is a transcription, that is the primer-dependent synthesis of the mRNA initiated by viral polymerase (Palese & Shaw, 2007). Primers necessary for the transcription reaction are generated by the endonuclease activity of the PB2 protein, whereas the synthesis of mRNAs is catalyzed by PB1 subunit of the polymerase (Julkunen *et al*., 2001). Then, mRNA chains are transported into the cytoplasm where the synthesis of early viral proteins essential for RNA replication occurs (Julkunen *et al*., 2001; Resa-Infante *et al*., 2011).

The newly synthesized PB1, PB2, PA, NP, and NS1 proteins are imported into the nucleus and take part in the regulation of the replication and secondary transcription process (Julkunen *et al*., 2001; Neumann *et al*., 2009). In the replication of the virus genetic material, a full-length copy of the viral RNA defined as complementary RNA (cRNA), is synthetized (Julkunen *et al*., 2001; Palese & Shaw, 2007). The cRNA molecules are the template for the production of the new viral RNAs (vRNAs), and in turn they are the template for the secondary mRNA synthesis (Julkunen *et al*., 2001). The synthesis of the mRNA, cRNA and vRNA is catalyzed by the viral polymerase, with various functions of each subunit engaged in different reaction steps (Neumann *et al*., 2004). After secondary transcription, mRNAs are decoded and production of all viral structural proteins occurs. Proteins, that are included in the RNP complex and also M1 and NS2 are transported into the nucleus. There, the assembly of new RNPs take place and with the support of M1 and NS2 peptides they are exported at progeny virions budding site (Julkunen *et al*., 2001). Not only segmented structure of the IAV genome, but also genome packaging have influence on the reassortment process. It is suggested that each RNA molecule on either ends has a specific packaging signal and the process of RNA packaging is hierarchic (the PB2, PA, NP and M segments play more important role than the others). During the transport from nucleus RNA, segments are not exported separately (RNA–RNA interactions), but also not all eight segments are transported at once (Gerber *et al*., 2014). Interface of M1 protein being a part of RNP-M1 complexes with M2 protein initiates virus assembly. M2, as well as HA and NA protein synthesis occurs on membrane-bound ribosomes and these proteins are transported to the host cell membrane through the endoplasmic reticulum and Golgi apparatus. The budding of progeny virus and the last step of releasing them from the surface of infected cell occurs through the sialidase activity of NA (Reperant *et al*., 2012) (Fig. 2).

REASSORTMENT — PANDEMIC EVENTS

Due to the genome structure of IAVs, during co-infection of a host cell with two or more IAV strains, exchange of their RNA segments may occur (Lu *et al*., 2014). Also, the fact that during the transcription and replication, each RNP works as an independent functional unit and increases the chance of gene reassortment (Resa-Infante *et al*., 2011). With eight RNA segments, there are 256 probable combinations for genes from two IAVs to shuffle. When IAVs exchange the HA and/or NA genes, an antigenic shift occurs. Reassortment is a common and important process in the IAV evolution and in crossing of the species barriers (Taubenberger & Kash, 2010). Through reassortment, novel IAVs acquiring new features can be created. Newly combined virus may have great pandemic potential in humans. As in the past, apart from the Spanish flu in 1918, all pandemics were associated with IAV reassortants. For both pandemics, H2N2 in 1957 (Asia) and H3N2 in 1968 (Hong Kong), reassortants of avian and human IAV were responsible (Lu *et al*., 2014). The H2N2 virus from 1957 was a lineal descendent of the H1N1 pandemic virus emerged in 1918, that had three segments of avian virus origin (the second — the PB1 gene, fourth — the HA gene and sixth -— the NA gene). In this case, antigenic shift from H1N1 to H2N2 occurs. Over the next 11 years, this virus was endemic. In 1968, H2N2 was even-

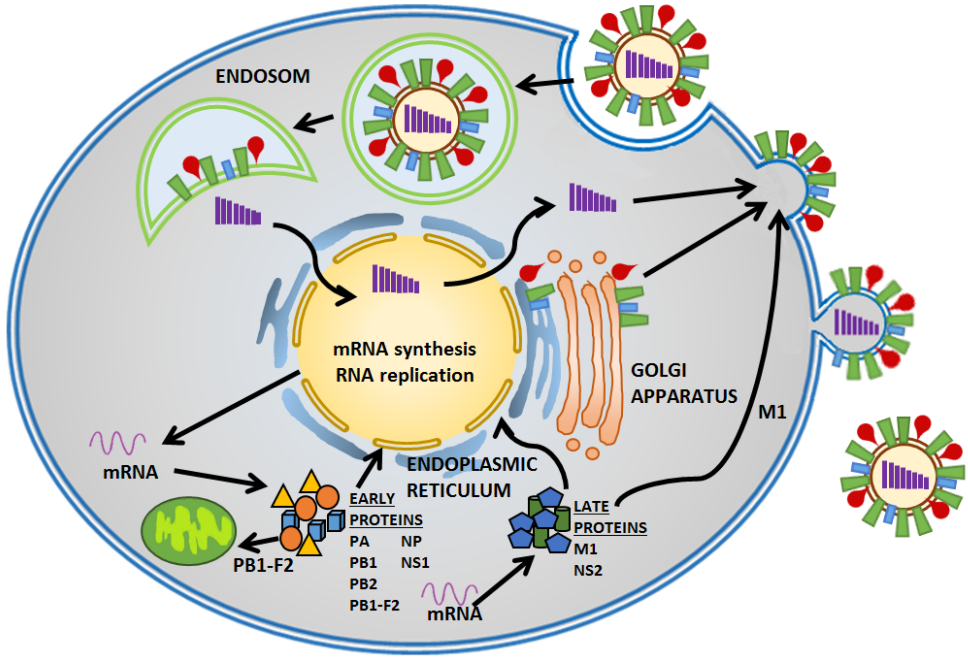

Figure 2. The IAV life-cycle.
The IAV binds to the receptors on the cell surface *via* the HA glycoproteins followed by its endocytosis. Then, viral and endosome membranes fuse and the genetic materials (RNPs) are realised into the cytoplasm. Then, RNPs are imported into the nucleus, where primary transcription occurs resulting with the synthesis of messenger RNA followed by the synthesis of early protein (PA, PB1, PB2, NP, NS1). These proteins are transported to the nucleus and are involved in replication, and secondary transcription. Novel viral RNA and late structural proteins are synthetized. The progeny virions are assembled and released from the cell (Neumann *et al.*, 2009).

tually replaced by another reassortant, which was the cause of the pandemic in Hong Kong. This new virus contained all gene segments from human H2N2 pandemic virus, except of HA (new H3 subtype) and PB1. The HA and PB1 gene segments were replaced by the novel avian-like segments (Taubenberger & Kash, 2010; Tscherne & García-Sastre, 2011). Also, H3N2 virus became seasonally endemic after the outbreak. The virus of 1957 disappeared entirely, whereas the H3N2 has been still circulating worldwide (Taubenberger & Kash, 2010).

Also in the recent pandemic in 2009, H1N1 reassortant played a crucial role (Lu *et al.*, 2014). This virus possessed gene segments from an avian IAV source (the PB2 and PA), the human seasonal H3N2 IAV (the PB1) and swine IAV lineages origin ('classical' swine IAV — the HA, NP and NS; the European avian-like IAV — the NA and M) (Taubenberger & Kash, 2010; Tscherne & García-Sastre, 2011).

IAVS ENDEMIC IN PIGS

Pigs are thought to be a mixing vessel of IAVs and play a significant role in their genetic reassortment. Swine population may became a reservoir for another lineage of IAV that can be introduced worldwide and constitute a great risk. The pig is the only domesticated mammal that is susceptible to all avian and human IAVs (Brown, 2000).

Nowadays, in pig population, IAVs of H1N1, H3N2 and H1N2 subtypes occur endemically all over the world (Brown, 2000).

In Europe, the H1N1 IAV of avian origin emerged in 1979 to pig population, replacing 'classical' swine H1N1. Five years later the reassortment of avian-like H1N1and human seasonal H3N2 viruses gave rise to the new human-like swine H3N2 virus lineage. In 1994, another reassortant emerged to swine population. This triple reassortant, human-like swine H1N2 virus possess gene segments from avian-like swine H1N1 and seasonal human H1N1 and H3N2 viruses (Zell *et al.*, 2013). In Poland up to 2008 most of the obtained swine IAV belonged to avian-like H1N1 subtype (95.2%). Only 2 swine human-like H3N2 viruses were isolated (4.8%). In 2011 the first Polish swine human-like H1N2 virus was obtained (Kowalczyk *et al.*, 2010; Kowalczyk *et al.*, 2012), and in 2012 the first Polish viruses of H1N1pdm09 were detected (Markowska-Daniel *et al.*, 2013).

In East and Southeast Asia, a circulation of all three IAVs subtypes in pig population is present (Brown, 2000; Poonsuk *et al.*, 2013). In China, up to 2002 'classical' swine H1N1 viruses were mostly isolated. European avian-like swine H1N1 and triple reassortant swine H1N2 were detected from 2001 and 2002, respectively. Over a period of three years since 2002 all mentioned above viruses as well as H3N2 co-circulated in pigs, but in 2005 the European avian-like swine H1N1 became dominant (Vijaykrishna *et al.*, 2011).

In the United States, up to 1998 only 'classical' swine IAV H1N1 was circulating in swine population (Ducatez *et al.*, 2011). Then, human H3N2 virus was introduced, resulting in a new IAV double and triple reassortants emerge (Poonsuk *et al.*, 2013). Double reassortant possessed the HA, NA and PB1 from human IAV and the other genes from 'classical' swine IAV, whereas triple reassortant contained genes of avian (the PA and PB2), human (the HA, NA and PB1) and 'classical' swine (the M, NP and NS) origin (Ducatez *et al.*, 2011; Poonsuk *et al.*, 2013). After the emergence of these viruses, many different reassortants appeared (Ma *et al.*, 2010; Ducatez *et al.*, 2011; Poonsuk *et al.*, 2013). Both swine H1N1 and

H3N2 subtypes circulating in the US are genetically different from those in Europe (Zell *et al.*, 2013).

The pandemic IAV H1N1 appeared in Mexico in 2009 and spread quickly worldwide (Ducatez *et al.*, 2011; Tscherne & García-Sastre, 2011; Vijaykrishna *et al.*, 2011). Shortly after the emergece of this virus, the cases of reassortment with endemic swine IAVs were reported in pigs globally (Vijaykrishna *et al.*, 2010; Ducatez *et al.*, 2011; Moreno *et al.*, 2011; Starick *et al.*, 2011; Tremblay *et al.*, 2011).

REASSORTMENT CASES

The reports of the emergence of new reassortants worldwide were noticeable continuously.

Castrucci and coworkers (1993) analysed phylogenetically Italian IAV strains circulating in swine population. They discovered the presence of internal protein genes from avian-like swine H1N1 in human-like swine H3N2 IAVs isolated since 1985. Before 1983 such reassortment has not been detected, thus the exchange of genes occurred between 1983 and 1985. These results support the hypothesis of pigs being a mixing vessel of IAVs.

Shin and coworkers (2006) reported the emergence of a new H3N1 IAV isolated from pigs, which is a reassortant of human-like H3N2 (HA gene) and swine IAV circulating in Korea (the other seven genes).

Cong and coworkers (2007) in their report characterized antigenically and genetically swine H9N2 IAV isolates. These viruses detected in northern China are reassortants of avian H9N2 and H5N1 IAVs. The HA and NA genes were found to be closely related to early chicken H9N2 virus identified in 1998, whereas the other genes were derived from H5N1 IAV.

In the central US, the isolation of H2N3 reassortant virus from two pig farms was reported by Ma and coworkers (2007). The HA, NA and PA genes segments of those IAV isolates derived from avian resources (H2N3 mallards isolates, H4N3 blue-winged teal isolates and H6N5 mallards isolates, respectively), whereas the other genes originate from contemporary in the US triple reassortant swine IAVs. Ma and coworkers (2007) reported that this virus replicates in pigs, ferrets and mice efficiently (models of human infections) and transmits among pigs and ferrets. Thus, this IAV carries a potential pandemic risk due to its ability to replicate efficiently in tested mammalian hosts.

REASSORTMENT EXPERIMENTS

The reassortment is an important mechanism of IAV evolution, which may lead to the emergence of new pandemic strains. This topic was of a great interest of many researchers all over the world. Among IAVs, avian H9N2 and H5N1 viruses are considered as potential causing agents of the next human influenza pandemic. These viruses, as well as pandemic H1N1/2009 IAV, coexist in human and pigs that provides an opportunity to exchange of genes (Sun *et al.*, 2011).

Several experiments focused on the reassortment process between different IAV strains were conducted (Chen *et al.*, 2008; Li *et al.*, 2010; Octaviani *et al.*, 2010; Zhang *et al.*, 2010; Sun *et al.*, 2011). Sun *et al.* (2011) studied 127 different reassortants of avian H9N2 IAV and pandemic H1N1/2009 IAV. They have found that these viruses have a high genetic compatibility. The percentage of reassortant replication ability was high (approximately 57.5%). Moreover, they confirmed that generated reassorants from avian H9N2 IAV and pandemic H1N1/2009 IAV could be more virulent than parental viruses.

Zhang and coworkers (2010) showed that also reassortment of highly pathogenic avian H5N1 virus and pandemic H1N1 viruses can result with a virus of a high infectivity. Similar results were obtained by Octaviani and coworkers (2010). They co-infected MDCK cells with pandemic H1N1 virus of swine origin and avian H5N1 virus and confirmed their high genetic compatibility. As regards, they observed that some reassortants showed a better growth than parental viruses.

Chen and coworkers (2008) and Li and coworkers (2010) studied reassortants of avian H5N1 and seasonal H3N2. Chen *et al.* (2008) analyzed viability, replication efficiency and virulence of 63 reassortants of H5N1 subtype. In this study, almost half of the reassortants replicate efficient in MDCK cell culture and 13 of all tested reassortans at dose $<10^4$ pfu were lethal for mice. However, none of their reassortant viruses was characterized by a higher virulence than parental H5N1. On the other hand, Li and coworkers (2010) tried to generate reassortants between low pathogenic avian H5N1 and human H3N2. They have managed to obtain 254 reassortants and more than 70% of them have different replicative abilities. To determine the pathogenicity, they used 75 reassortants from which 44, 9 and 22 strains have lower, similar and higher pathogenicity than the H5N1, respectively. Li and coworkers (2010) indicated that the presence of human PB2 gene segment in avian H5N1 virus increases its virulence.

These findings support the idea that reassortment between avian and human/swine influenza viruses may result in emerging IAV strains with great pandemic potential.

CONCLUSIONS

New IAVs resulting from the reassortment process appeared continuously. Up to now, three pandemics were caused by avian/human and/or swine reassortant.

In order to achieve a more accurate prediction and response to emergence of novel IAV, it is necessary to gain an insight in the characteristics of the IAVs and improve the knowledge on the gene constellation. Therefore, in order to minimize the risks of reassortment and introducing of novel pandemic IAVs, a continuous surveillance and containment measures on global scale are essential.

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