Evolutionary dynamics of highly pathogenic avian influenza A/H5N1 HA clades and vaccine implementation in Vietnam

Based on hemagglutinin (HA) and neuraminidase (NA), influenza A virus is divided into 18 different HA (H1 to H18) and 11 NA types (N1 to N11), opening the possibility for reassortment between the HA and NA genes to generate new HxNy subtypes (where x could be any HA and y is any NA, possibly). In recent four years, since 2010, highly pathogenic avian influenza (HPAI) viruses of H5N1 subtype (HPAI A/H5N1) have become highly enzootic and dynamically evolved to form multiple H5 HA clades, particularly in China, Vietnam, Indonesia, Egypt, Cambodia, and Bangladesh. So far, after more than 10 years emerged in Vietnam (since late 2003), HPAI A/H5N1 is still posing a potential risk of causing outbreaks in poultry, with high frequency of annual endemics. Intragenic variation (referred to as antigenic drift) in HA (e.g., H5) has given rise to form numerous clades, typically marking the major timelines of the evolutionary status and vaccine application in each period. The dominance of genetically and antigenically diversified clade 2.3.2.1 (of subgroups a, b, c), clade 1.1 (1.1.1/1.1.2) and re-emergence of clade 7.1/7.2 at present, has urged Vietnam to the need for dynamically applied antigenicity-matching vaccines, i.e., the plan of importing Re-6 vaccine for use in 2014, in parallel use of Re-1/Re-5 since 2006. In this review, we summarize evolutionary features of HPAI A/H5N1 viruses and clade formation during recent 10 years (2004-2014). Dynamic of vaccine implementation in Vietnam is also remarked.

Keywords: Orthomyxoviridae, Subtypes, HPAI A/H5N1, Genotypes, Clades, Reassortment, Vaccines, Vietnam

Introduction

Avian influenza is an acute infectious disease that occurs and spreads very fast in poultry, waterfowls, wild birds, animals, and able to zoonotically be transmitted to humans. Avian influenza is listed as one of the most dangerous diseases by World Organisation for Animal Health (OIE; http://www.oie.int/eng/normes/mmanual/A_summry.htm) that a special attention is needed for control. Influenza A virus belongs to the family of Orthomyxoviridae, of which the envelope is embedded by two surface glycoproteins, e.g., hemagglutinin (HA) and neuraminidase (NA). The virus has a spherical appearance with a diameter of 80-120 nm, or polymorphological shape, sometimes is elongated, like fibers and arranged in a sequence. Virus particles (virions) have a simple structure, which includes envelope, capsid and a core containing genetic material (viral genome) with molecular weight of about 250 million daltons [1]. Influenza A virus
genomes contain a single-stranded negative RNA ([-ssRNA]) of 8 segments (polymerase basic 2 [PB2], polymerase basic 1 [PB1], polymerase acid [PA], HA, nucleoprotein [NP], NA, matrix [M], non-structural polypeptides [NS]) [1,2], encoding for 15 proteins in overall, including PB2, PB1, PA, HA, NP, NA, M1, M2, NS1, NS2, and supplementary proteins recently identified with little or unknown functions [3], i.e., PB1-F2 [4], PB1N40 [5], PA-X [6], PA-N155 and PA-N182 [7]. Up-to-date, there are 18 HA and 11 NA types for influenza A viruses to be known, based on the composition and activity of the HA (from H1 to H18) and the NA (from N1 to N11) genes, especially, by recent addition of two new HA (ie., H17 and H18) and two new NA types (N10 and N11) detected in bats [8,9]. The first 15 HA and 9 NA types are originally found in aquatic fowles which served as a source for subsequent reassortment [3]. The numerous types of HA and NA enable the components going through genetic reassortments between HA and NA for generation of new subtypes [10-12].

Major polypeptides encoded by 8 segments of the genome of the influenza A virus are as follows:

1) The polymerase complex (PB2, PB1, and PA): The polymerase enzymes encoded by segments 1, 2, and 3, respectively, are responsible for transcription and replication of viral RNA [13]. Additionally, the recently identified polypeptides of PB1-F2, PB1-N40, PA-X, PA-N155, and PA-N182 encoded within segments 2 and 3, respectively, have confirmed the influenza virus as a top of the most complicated viral agents by multi-disciplinary utilization of the genome and the functional polypeptides [3].

2) The surface antigenic proteins (HA and NA): HA assists virus in attachment to the cell receptors; and by being cleaved by protease, facilitating intrusion of viral genetic material into the cell. NA functions in promotion of the assembly and the release of the virus progenies from the cell receptor [14]. The HA and NA antigenic glycoproteins determine the specificity of different subtypes of influenza A viruses and NA is a target for antiviral treatment [15]. Moreover, HA and NA also play a pivotal role in determining the antigenicity of vaccine production [11,14,16].

3) The proteins encoded by internal genes: They include NP, M (M1 and M2), and NS (comprising NS1 and NS2): Products of these genes play important roles in packaging of viral genomic RNA during replication, in intracellular transporting pathways and in shipping mechanism through the channel components [17,18]. M1 protein sustains the virion structure, playing multiple role in the replication, assembly, and release of viruses [1]. The viral ribonucleoprotein complexes (vRNPs) contain viral RNA, viral polymerases and viral NPs which are essentially formed during viral infection and replication [19, 20]. NS2 product also known as a nuclear export protein or NEP is essential for transportation of the vRNPs to cytoplasm [1].

After more than 10 years of highly pathogenic avian influenza (HPAI) A/H5N1 emergence in Asia, the reassortments of HAs and NAs have always occured to generate new subtypes [21,22]. Just in a short time of recent 5 years, more lethal, pandemic candidate and/or pandemic subtypes of influenza A viruses have been reassorted and emerged in wide geographical localities [3]. These are represented by, namely, the H5N1 variants of highly differentiated clades, the pandemic A/H1N1-2009, the new subtypes of H7N9 and H10N8 in China that posed a high risk across different nations [23]. The rapid emergence of the antigenic drifted HPAI H5N1 and the newly reassorted H7N9 and H10N8 viruses raise the urgent needs to definitely update vaccine development from antigenicity-matching strains for actual application [15,24-26]. Fortunately, a great technology, reverse genetics has contributed at a great speed, to solve the vaccine development in the shortest time, that new vaccine(s) against most recently emerging subtypes, with high immunity and warranted protection, including the H5N1 and H7N9 vaccines have been generated [25-30].

In this review, we focus on the recently-introduced HA evolution, preferably, on H5 HA of HPAI A/H5N1 strains and the evolutionary features of clade formation in Vietnam, by 1) summarizing the antigenic drift of H5 HA and the emergence of its clade/subclades in the past eleven years, since 2003; 2) analyzing phylogenetic relationships of different HA clades between Vietnam and neighbouring countries; 3) looking at spatial-temporal transmission of clades and dynamic vaccine implementation in Vietnam. Literature has been taken for use mainly from the international sources but in some cases, from some of local publications with actual citations.

**Evolutionary Features for Emergence of Genotypes and Clades of H5N1 in Vietnam**

It is noted that the precursor highly pathogenic A/H5N1 avian virus was first time formed in 1996, as a result of the genetic reassortment of the H5 HA and the N1 NA types in wild geese in Guangdong, China (A/Goose/Guangdong/1/96[H5N1]); and the reassortants subsequently derived from this ances-
The H5N1 strains isolated in Vietnam during 2004-2006, were of the Guangdong sublineage, belonging to the genotype Z. Recently, the additional genotype G has emerged during 2007-2008; and after 2010, both of Z and G genotypes have coexisted up to date [40,43]. Two major groups of subgenotypes have been found to exist in Vietnam, subgenotype N (North) predominant in the North and S (South) in the South [36,41]. Between 2007 and 2010, it was noted that the circulating strains were of mixed sublineages, i.e., Guangdong-like, Fujian-like and Qinghai-like co-existed [41-43].

In the recent 10 years, the H5 antigenic gene has high tendency to drift, generating complicated intragenic changes. As results, the newly emerged variants have enhanced their adaptability to multi-host transmissions, helping the HPAI A/H5N1 viruses to spread throughout the world; hence the level of pathogenicity has been exacerbated [32,34]. Since 1996, during 8 years by 2004, H5N1 has diversified to generate 10 distinct clades (0-9) due to the antigenic mutational drift in the H5 gene, all of which have always originally first time formed in China [3]. The emergence of the HPAI A/H5N1 viruses in early 2000s has accelerated the H5 gene undergoing onto dynamic evolution in a high speed, generating numerous new clades/subclades of the second-, third-, and forth-order subclades, mostly within clades 1 and 2 [3,44]. Not all the above mentioned clades have been introduced into Vietnam (Fig. 1) [41-46], but largely, the rooting clades have further diversified to form new serial-order subclades, heavily affecting the avian influenza occurrences in poultry and wild birds, and complicating the epidemiologic situation in Vietnam [40,42,44,47-49].

By 2014, for example, clade 2 has diversified into different subclades and expanded over the world. The 2.3.4 clade alone evolved to the forth order subclades of 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.4.4, 2.3.4.5, 2.3.4.6 over China [50] of which the subclades 2.3.4.1, 2.3.4.2, 2.3.4.3 have emerged and existed in Vietnam for a period of 4 years, during 2007-2010 (Fig. 1). The newly emerged clade 2.3.2 appeared more expandable in Vietnam, Southeast Asia, North Asia and many countries in Eastern Europe from 2009 to 2014, widely detected in wild birds, then poultry in China (Qinghai lake region), Mongolia, Russia, Japan, Korea, Laos, Hong Kong, Vietnam, Japan, Bangladesh, India, Bulgaria and recently, in Indonesia [42,51-58]. These strains are now classified into forth and fifth-order clades with an additional letter of the right-most digit implemented [44].

Major clades, such as 2.1.3, 2.2.1, 2.3.2, have also undergone substantial evolution in HA gene, resulting in a deep diver-
fication, even within clade 2.3.2.1, there have been novel endemic, dynamically differentiated variants of a, b, c subgroups, during 2010-2014 in Vietnam. These 2.3.2.1a/b/c viruses, are preferably circulating in waterfowles, sometimes with no symptoms, thus are confusingly affecting the vaccination programs as well as complicating the epidemiological situation in a number of countries in the region [42,43,58,59]. Although six clades have been found among the A/H5N1 strains isolated since 2003 in Vietnam, including 1, 2, 3, 5, 7, 8, only three (clades 1, 2 and 7) have rooted to spatially/temporally last and to monophyletically diversify into discrete subclades of one-, two-, three- or forth-order designations (Fig. 1). These clades of 1, 2, 7 and their digit number-order subclades seemed to undergo on-in-depth H5 based-drifting for formation of the further fifth-order groups, e.g., in case of clade 2.3.2.1a/b/c, officially nomenclatured by adding alphabetical implementation [44]. The derivative strains of these clades are firmly now circulating in multiple species of gallinaceous poultry throughout Vietnam [42,43,48,60].

**Fig. 1.** Emergence timelines of hemagglutinin-based clades of A/H5N1 in Vietnam (only those clades that emerged and identified in Vietnam are presented). Data used for making timelines of H5N1 occurrence were collected from the following published sources: Nguyen et al. [41,42], Creanga et al. [43], World Health Organization/World Organisation for Animal Health/Food and Agriculture Organization (WHO/OIE/FAO) H5N1 Evolution Working Group [44], Inui [45]; A/B/C marks for clades were taken from the above listed publications; question mark (?) indicates upcoming confirmation of clades in 2014.

**Diversification of the Predominant Clades (1, 1.1, 2.3.4, 2.3.2.1 and 7, 7.1, 7.2) in Vietnam**

Approaching 18 years from the time of first generation, the Gs/Guangdong lineage-H5N1 avian influenza virus reasor-
vaccines (clade 2.3.4 origin) imported from China were in use and a national project of testing NIBRG-14 masterseed (rgA/Vietnam/1194/2004, clade 1, obtained from National Institute for Biological Standards and Control [NIBSC, UK]), was launched for vaccine production [61,62]. In this period, the first detection of a newly designated clade 7 at ports of entry in Lang Son province was published [60,63].

4) In 2010: This year was the time for the new clades emerged, and alternatively, for the appearance of periodically existed viruses [42]. The emergence of newly introduced clade 2.3.2 and further evolution of clade 2.3.2.1 into 2.3.2.1a/b was noted in the North; and the continual evolution of clade 1.1 of the Cambodian lineage into 1.1.1 and 1.1.2 occurred in the South [42,43,47,64]. Re-1 and Re-5 vaccines were in use.

5) During 2011-2012: The dynamic formation of a, b, and particularly, c subgroups of clade 2.3.2.1 was in progress in whole country [43,65]; and because of antigenicity-matching failure for clade 2.3.2.1b, the Re-1/Re-5 vaccination was ceased in some provinces in the North [66]. NIBRG-14 based vaccine (namely, NAVET-Vifluvac) was successfully tested, indicating reliable efficacy against viruses of the current clades, particularly when used with immunomodulating adjuvants like β-glucan [61,62,67]. Clades 1.1.1/1.1.2 were in complete dominance in the South and Re-1 vaccine was still in use [47,49].

6) During 2013-2014: There was a wide circulation of clade 2.3.2.1a,c in whole country and no report of clade 2.3.2.1b was made anymore. Concurrently, clades 1.1.1/1.1.2 were heading from the Mekong Delta region to Central Vietnam. This time, the re-recognition of clade 7.1/7.2 evolving from clade 7 was reported. Due to decline in protection and wide spread of clade 2.3.2.1c, Re-6 vaccine is planned to import for use in 2014 [49]; according to the Vietnam authority, Department of Animal Health, 2014 (http://www.cuchuy.gov.vn/).

Tracing back the original source of H5N1, it is indicated that all viruses of the pre-existing clades 2.3.4, 2.3.2, 1.1, 7 before 2010; and the current circulating 2.3.2.1a/(b)/c, clades 1.1.1/1.1.2 and clades 7.1/7.2 in Vietnam have been introduced from Southern provinces of China during 2003-2014 [43]. It should also be noted that the very virulent clade 1.1.1/1.1.2 viruses currently dominating the South of Vietnam have evolved from a Cambodian sublineage of clade 1.1 [3]. To examine the clustering of viruses in different clades using World Health Organization (WHO)/OIE/Food and Agriculture Organization of the United Nations (FAO)-2014-nomenclaturing system, we include those previously listed as references for clading source and the current sequences obtained by our study from samples collected during 2008-2014. The phylogenetic tree resulting from H5 nucleotide data (using 77 sequences comprising of representative clades) is shown in Fig. 2. It clearly shows that most recent 2009-after collected isolates of Vietnam are completely clustered with the H5N1 HPAI strains of newly described clade 2.3.2.1 (and of its clade 2.3.2.1a/b/c) emerged after 2009, closely linked to A/Hubei/1/2010 (clade 2.3.2.1a), to A/barn-swallow/Hong Kong/1161/2010 (clade 2.3.2.1b) and A/Hong Kong/6841/2010 (clade 2.3.2.1c), respectively.

Strains forming clade 2.3.2.1a and clade 2.3.2.1b, comprised HA sequences (majority are of our study, be included in analysis) which were closely linked to A/Hubei/1/2010 and A/barn-swallow/Hong Kong/1161/2010, respectively. Clade 2.3.2.1c viruses, all clustered together, around the reference A/Hong Kong/6841/2010 strain, are of some typical characteristics of taxonomic grouping. It is indicated that the old strains, originally isolated in 2009 at Qinghai lake (China), or those before 2009 including the ancestral A/BHG/Qinghai/1/2009 (accession No. HQ020367) [68] formed a basal branch, formerly named as clade 2.3.2, now classified as clade 2.3.2.1c (Fig. 2). The tree shown in Fig. 2 supported a precise topology of clade 2.3.2.1c strains, with the Vietnamese (of those isolated in 2014 of our study as well) and Indonesian viruses [58] in two separate groups. Distinct from clade 1 they have evolved from, all HA sequences of clades 1.1.1/1.1.2 performed a monophyletically close group, regardless of where they were identified from isolates, in Vietnam or Cambodia (Fig. 2).

Antigenic Characteristics of A/H5N1 Viruses in Vietnam

Since their re-emergence in 2003, HPAI A/H5N1 viruses have become enzootic in some countries and continue causing outbreaks in poultry as well as sporadic human infections. The A/H5N1 viruses have diversified both genetically and antigenically leading to the need for multiple vaccine development. Evolution of the HA gene of influenza A/H5N1 viruses emerged in Vietnam has marked major antigenic changes, affecting immune response in the vaccinated poultry [42,47].

Amino acid analysis of H5 HA of the emerging strains isolated during 2010-2013 in Vietnam has revealed that the polybasic cleavage site (amino acid 338-345, H5 numbering) of these viruses, was similar to the H5 HA belonged to viruses of clade 2.3.2.1 and its subgroups (2.3.2.1a,b,c) recently re-
ported in China, South Korea, Japan, Mongolia, Nepal, Laos, Russia, Bangladesh and Indonesia [3,58,69,70]; and similar to those of clade 2.3.2 origin, but different from clade 2.3.4 and clade 1 (and 1.1) (Table 1) [3,42,43]. The strains found in South of Vietnam have evolved from clade 1, resulting in subclades 1.1.1 and 1.1.2, similar to that of the Cambodian clade 1.1 [3,44,70]. However, amino acids of receptor binding site of strains (QSG, position 238-240) in all clades in Vietnam and elsewhere were conserved (Table 1), keeping the ability of binding specificity stable throughout the evolution of HA [14,59].

Analysis of amino acid identity at the antigenic sites (Table 1) revealed that all strains of clade 2.3.2.1a/b/c have amino acid K at antigenic position 69; ANPA at 99-102 (for some strains, N was replaced by K or S); and a highly conserved sequence, DHEASL, at 140-145 (antigenic site 1) except for the Vietnamese clade 2.3.2.1c strains of 2014 who possess NHEVSL.

Glycosylation at amino acid 170 in HA (position 170-172) is completely lost by changing from NST (as seen in clade 1 and 1.1 strains) and NNT (clade 2.3.4) to amino acids DNA in all strains of clades 2.3.2 origin and current 2.3.2.1(a,b,c) of Vietnam, China, Bangladesh, Indonesia and other countries [43,59,71], including those strains collected in 2014 of our current study (Table 1).

Spatial-Temporal Transmission of H5N1 Clades and Dynamic of Vaccine Application in Vietnam

Clade 2.3.2.1 and clade 1.1 (now 1.1.1/1.1.2) have revealed a great diversification due to their complex evolution of HA and endemically spatial transmission after 2010 in Vietnam. Clades 7 and 7.1/7.2 were temporarily detected before and now re-emerged in some places.

These evolutions and transmissions are characterized by the next descriptions, as follows:

1) Viruses of clade 2.3.2.1a (phylogenetically designated as A/Hubei/1/2010[H5N1]-like), emerged first time in mid-2010
Table 1. Amino acid residues at sites of protease cleavage, receptor binding, epitopes of antigenic sites and a site of variable glycosylation of the H5 hemagglutinin polypeptide

| Virus strains                  | Country/Territory | Clade | Cleavage site        | Receptor binding site | Antigenic sites | Glycosylation change |
|-------------------------------|-------------------|-------|----------------------|-----------------------|-----------------|---------------------|
| A/Dk/VN/HT5 (2014)           | Vietnam           | 2.3.2.1c | 338-345/346          | 238-240               | 69              | 140-145 Site 1      | 152-157 Site 2      | 170-172            |
| A/Dk/VN/KH23 (2013)          | Vietnam           | 2.3.2.1c | QRERRRK-RG           | QSG                   | K               | ANPA               | NHEVSL             | SYQGNS             | DNA                |
| A/Dk/Tegal/1727-11 (2012)    | Indonesia         | 2.3.2.1c | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/BHG/Qinghai/1 (2009)       | China             | 2.3.2.1c | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/HK/6841 (2010)             | Hong Kong         | 2.3.2.1c | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Mdk/VN/LBM66 (2011)        | Vietnam           | 2.3.2.1b | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Bam-sw/HK/1161 (2010)      | Hong Kong         | 2.3.2.1b | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Dk/VN/LBM136 (2012)        | Vietnam           | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Ck/VN/TH2c1 (2012)         | Vietnam           | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Dk/VN/07801 (2011)         | Vietnam           | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Hubei/1 (2010)             | China             | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Mall/GOO/KO/10-1515 (2011) | South Korea       | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | AKPA               | DHEASL             | SYQGNS             | DNA                |
| A/Phalcon/Tochigi/15 (2011)  | Japan             | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | AKPA               | DHEASL             | SYQGNS             | DNA                |
| A/Grebe/Grebe/1/2009         | China             | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/BHG/Mongolia/X53 (2009)    | Mongolia          | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Dk/NP/81 (2010)            | Nepal             | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Dk/LA/469 (2010)           | Laos              | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Grebe/Grebe/1/2009         | Russia            | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | AKPA               | DHEASL             | SYQGNS             | DNA                |
| A/Portugal/1/2009            | Hong Kong         | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Bcn/Hermon/HK/659 (2008)   | Hong Kong         | 2.3.2.1a | QRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Guangxi/1 (2008)           | China             | 2.3.4.3 | LRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/VN/HN3124/2 (2007)         | Vietnam           | 2.3.4.3 | LRERRRK-RG           | QSG                   | K               | ANPA               | DHEASL             | SYQGNS             | DNA                |
| A/Ck/VN/NCVD/093 (2008)      | Vietnam           | 7.2    | QRERRRK-RG           | QSG                   | K               | ASPA               | NHETSL             | SYLENP             | NNT                |
| A/Ck/VN/NCVD/016 (2008)      | Vietnam           | 7.1    | QRERRRK-RG           | QSG                   | K               | ASPA               | NHETSL             | SYLENP             | NNT                |
| A/Mdk/VN/OIE-0043 (2012)     | Vietnam           | 1.12   | QRERRRK-RG           | QSG                   | R               | ANPV               | SHEASL             | SYQGNS             | NST                |
| A/Mdk/VN/OIE-559 (2011)      | Vietnam           | 1.11   | QRERRRK-RG           | QSG                   | R               | ANPV               | SHEASL             | SYQGNS             | NST                |
| A/Kh/0445/050 (2007)         | Cambodia          | 1.1    | OREGRRRK-RG          | QSG                   | R               | ANPV               | SHEASL             | SYQGNS             | NST                |
| A/NA/1194 (2004)             | Vietnam           | 1      | QRERRRK-RG           | QSG                   | K               | ANPV               | SHEASL             | SYQGNS             | NST                |
| A/NA/1203 (2004)             | Vietnam           | 1      | QRERRRK-RG           | QSG                   | K               | ANPV               | SHEASL             | SYQGNS             | NST                |
| A/Gs/Guangdong/1 (1996)      | China             | 0      | QRERRRK-RG           | QSG                   | R               | ASPA               | NHDASS             | SYHGRS             | NSA                |

Bold letter of amino acid indicates a change in comparison to other strains of same clade.

Dk, duck; Ck, chicken; BHG, black headed gull; HK, Hong Kong; Mdk, muscovy duck; VN, Vietnam; sw, swallow; MalDk, mallard duck; KR, South Korea; KH, Cambodia; Gs, goose.

*Viruses from our own study.

†Prototype reference strains for clade 2.3.2.1a/b/c.

in some provinces of the North of Vietnam. During the past four years since then, HA 2.3.2.1a viruses spatially spread over the coastal provinces along Central Vietnam, reaching Mekong Delta Basin, and currently (early 2014) predominantly are present in whole country [42,44]. For vaccination of poultry, Re-5, an imported vaccine from China for use in Vietnam, also known as Harbin Re-5 (reverse genetics-based A/duck/Anhui/1/2006 of HA clade 2.3.4 antigens), produced by the Harbin Veterinary Research Institute, Heilongjiang province, China, is still in nationwide use, showing reasonable efficacy for protection against clade 2.3.2.1a viruses [49]. The fact is that, circulating viruses of this 2.3.2.1a clade have not caused great loss to poultry and humans in Vietnam. Both Re-5 and Re-1 vaccines (Re-1, implemented since 2006; of rgA/Goose/Guangdong/1996 of HA clade 0, generated by reverse genetics) conferred a high level of protection (over 90%) in chickens against clade 2.3.2.1a by challenge test in laboratory; and about 70% seropositivity (by HA inhibition test) in immunized...
ed poulty in the field. However, protection level of Re-1/Re-5 vaccines, by challenging with strains of novel clade 2.3.2.1 (a, b, c), was much lower in ducks and muscovy ducks [47,49,65,67,69,73].

2) Viruses of clade 2.3.2.1b (A/barn-swallow/HK/1161/2010 [H5N1]-like), were first time identified in early 2011 in some localities in the North Vietnam such as Quang Ninh, Bac Ninh, Bac Kan, Thai Nguyen, Phu Tho, Nam Dinh, Thai Binh, Son La, Ninh Binh, Nghe An [42,43,64,66]. Up-to-date, viruses of this clade 2.3.2.1b circulated restrictly in China, Hong Kong SAR and Vietnam [44]. In Vietnam, clade 2.3.2.1b viruses seemed to limit their spatial and temporal transmission in the North of Vietnam. Nghe An province was the last geographic locality where the latest clade 2.3.2.1b was detected in 2012. In 2013, no further report has been made about the presence of HA clade 2.3.2.1b viruses, assuming that they have completely disappeared from the circulating A/H5N1 population in the country [44].

3) Viruses of clade 2.3.2.1c (the reference strain therein is A/Hong Kong/6841/2010[H5N1]), was first time identified in 2012, from outbreaks of chickens and ducks in several boundary northern provinces, then spread southbound to Quang Ngai, Khanh Hoa provinces of Central Vietnam. These viruses are now shifting towards the provinces in Mekong Delta of Southern Vietnam, and predominantly present in the whole country [43,66,67,70]. Thus, clade 2.3.2.1c is of a great challenge to HPAI A/H5N1 control in Vietnam, since the current in-use-vaccines showed rapid decline of protection against these viruses; and of course, presenting a high risk to public health [49]. Regarding to nationwide immunization of poultry, the current-in use Re-1 and Re-5 vaccines do not have sufficient efficacy to prevent the 2.3.2.1c clade viruses [49], requesting a new vaccine with higher matching of antigenicity to be implemented [43]. The Re-6 vaccine (rgA/dk/Guangdong/S1322/2010[H5N1] of clade 2.3.2), developed by the Harbin Veterinary Research Institute (China), has been imported for use in 2014, targeting the prevailing clade 2.3.2.1 in Vietnam.

4) Currently, clade 2.3.2.1 tends to spread in spatial distance from North to South of Vietnam, warning the vertical transmission along the country. Concurrently, strains of more virulent clade 1.1, now evolved into 1.1.1 and 1.1.2, are predominantly present in Mekong Delta provinces, which have been phylogenetically revealed to share close evolution of the Cambodian clade 1 progenitors [42,44,73]. The presence of clade 1.1 and its subclades 1.1.1/1.1.2 in several provinces of Central Vietnam in 2013, indicated that now these clades are heading towards North Vietnam from Mekong Delta Basin. The overlapping region for spatial transmission of clades 1.1/1.1.1/1.1.2 and 2.3.2.1a/b/c is lower Central Vietnam provinces; and interestingly, contrary orientation of HPAI A/H5N1 infections is likely of a consequence of free poultry trade due to North-South/South-North transportation, between provinces within Vietnam and between Vietnam and Cambodia [43,47].

5) Clade 7, 7.1/7.2 viruses (A/chicken/Shanxi/10/2006-like), representing an interesting evolution of clades in Vietnam, were on site detected in chickens seized at ports of entry in 2008 [60] and subsequently, viruses of these subclades (7.1/7.2) were identified in some places of Northern Vietnam [42,43,63]. The early introduced A/chicken/Vietnam/NCVD-016/2008 (No. FJ842476) was considered as an ancestral entry strain for diversification of the recently recognized clade 7.1 and 7.2 viruses.

Conclusion

From 2009 to date, in recent 5 years, a number of new antigenic subgroups of H5N1 have been formed mainly from two sources in Vietnam: newly introduced clades (2.3.2.1) from Southern region of China, and clade 1.1 localized in the Mekong Delta evolved from Cambodian lineages, heading to move in the opposite direction between the North and South Vietnam. Meanwhile, the in-depth diversified clade 2.3.2.1 viruses (with a, b and c monophyletic groups) have complicated the epidemiologic and vaccination status in the whole country. Because of failure in immune response in poultry vaccinated with Re-1 vaccine (clade 0) and Re-5 (clade 2.3.4) to viruses of the currently circulating clade 2.3.2.1b/c, the Vietnamese government has announced a temporary suspension of the vaccination program with these vaccines in some of the northern provinces. Significant amino acid substitutions in the strains of clade 2.3.2.1c emerged since 2011, were the main cause of the protective decline of the available vaccines currently used nationwide. The biased efficacy of the Re-1 and Re-5 imported vaccines has forced Vietnam to import Re-6 vaccine (clade 2.3.2) currently being used in 2014. The transmission tendency of two predominant clades, i.e., 2.3.2.1 (a,b,c) and 1.1/1.1.1/1.1.2, needs special works for active management of the endemics under control and containing enforcement of the virus spread.
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