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Comparision of genetic variation between avian influenza type A H5N1 virus causing disease and circulating on poultry in some provinces in the Mekong Delta in 2016

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ABSTRACT

The study is aimed to determine the genetic variation of type A H5N1 avian influenza virus in some provinces in the Mekong Delta in 2016. Oro-pharyngeal swab samples were collected on healthy chickens, ducks that were sold in markets and at slaughterhouses; tissue samples were also collected from poultry suspected cases of type A H5N1 avian influenza. These samples were tested by real time reverse transcription polymerase chain reaction (rRT-PCR) technique to detect type A H5N1 avian influenza virus. Hemagglutinin (HA) gene of some representative samples were sequenced to determine the genetic variation and virus clade. There were sequenced 10 HA genes of avian influenza type A H5N1 virus. The results of the genetic variation survey showed that the nucleotides homology rate between avian influenza type A H5N1 strains causing disease and circulating on poultry in the Mekong Delta provinces in 2016 were from 94.5% to 98.5% and amino acids were from 92.5% to 99.3%, respectively. The sequence of the amino acids at the linkage between the HA1 and HA2 fragment was RRRKR similar to highly pathogenic avian influenza virus as A/chicken/Korea/IC546/2011 and A/Hubei/1/2010 references. This indicates that the avian influenza type A H5N1 virus isolated in this study is highly pathogenic. Avian influenza type A H5N1 viruses circulating and causing disease on poultry in some provinces in the Mekong Delta in 2016 belong to the clade virus 2.3.2.1c.

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1 INTRODUCTION

Avian influenza is an acute infectious disease of several avian species, caused by the influenza A virus of the Orthomyxoviridae family. Influenza viruses are classified into two groups as highly pathogenic avian influenza and low pathogenic avian influenza. This classification is based on the pathogenicity of the avian influenza virus (OIE Terrestrial Manual, 2015). In the recent years from 2015 to 2017 and the early of 2018, there have been some outbreaks of avian influenza type A H5N1 in some provinces in the Mekong Delta. In addition, compared to some years ago, the rate of vaccination for avian influenza type A H5N1 in some localities was decreased because the vaccination system was gradually moving to socialization that increased the risk of occurrence of type A H5N1influenza outbreaks. The result of the study of Tien Ngoc Tien *et al*. (2016) showed that the prevalence of type A

H5N1 avian influenza virus in some provinces in the Mekong Delta in 2015 was 6.5%. Therefore, it can be shown that these areas have just been circulating the virus, and the disease outbreak occurred at the same time. This study was conducted to determine the genetic variation between type A H5N1 avian influenza viruses causing diseases and circulating on poultry in some provinces in the Mekong Delta.

2 MATERIALS AND METHODS

2.1 Materials

The study was conducted in An Giang, Ca Mau, Dong Thap, Can Tho, Soc Trang and Tra Vinh provinces from January 2016 to December 2016. Research subjects were healthy chickens and ducks sold in markets or in slaughterhouses; poultry have suspected clinical signs, lesions of type A H5N1 avian influenza.

2.2 Sampling method

Oro-pharyngeal swab samples were collected from healthy chickens and ducks that sold in markets and at slaughterhouses. Individual sample from 5 birds was pooled into one testing sample (OIE Terrestrial Manual, 2015). A total of 120 samples from ducks and 120 samples from chicken were collected in An Giang, Ca Mau, Dong Thap, Can Tho city (30 duck samples and 30 chicken samples per each province or city).

A total of 23 specimens (brain, spleen, treachea and lung) of poultry with typical symptoms of suspected cases of type A H5N1 avian influenza were collected (01 sample from Ca Mau, 10 from Can Tho, 11 from Soc Trang and 01 from Tra Vinh).

2.3 Testing method

All swab and tissue samples (brain, spleen, trachea and lungs) were taken to laboratories and tested by real time reverse transcription polymerase chain reaction (rRT-PCR) technique to identify type A H5N1 avian influenza virus. When samples positive for type A H5N1 avian influenza viruses were identified, the HA gene was sequenced to compare genetic variation by using the Molecular Evolutionary Genetics Analysis (MEGA 6.0; Tamura *et al.,* 2013).

2.4 Sequencing and analysis of the HA gene sequence to compare the genetic variation of type A H5N1 avian influenza virus method

Hemaglutinin (HA) of type A H5N1 virus was sequenced with two pairs of specific primers which were amplified by transcription polymerase chain reaction (RT-PCR). The first primer amplifies the HA1 gene fragment with an amplification size of 1,100 bp and the second primer amplifies the HA2 gene amplification size of 1,000 bp. The primer pairs were used in the study following the guidelines of the Centers for Disease Control and Prevention (CDC). Primers used in RT-PCR include:

Primers used in RT-PCR to obtain the HA1 gene fragment were:

Forward primer: 5 'AGCAAAAGCAGGGGTY-TAAT 3',

Reverse primer: 5 'CCATACCAACCATCTAY-CATTCC 3'.

Primers used in RT-PCR to obtain the HA2 gene fragment were:

Forward primer: 5'AYGCMTAYAAR-ATTGTCAAG 3 '

Reverse primer: 5 'AG-TAGAAACAAGGGTGTTTTTAAC TACAAT 3'.

Reagent composition of the master mix (Invitrogen Superscript III Platinum One step qRT-PCR Kit - US) as follows:

The volume of each reaction was 25 µl including water without enzyme destroying RNA and DNA: 18.75 µl; buffer solution (2x): 3 µl; forward and reverse primer (20µM): 2 µl; enzyme: 0.25 µl. The cycles were as follows: 1 cycle (50^{o}C for 30 minutes, 94^{o}C for 3 minutes) then 35 cycles (94^{o}C for 15 seconds, 60^{o}C for 45 seconds) and final cycle for 72^{o}C for 8 minutes (SuperScript ™ III Platinum ™ One-Step qRT-PCR Kit Product Information Sheet).

Amplification product measured by electrophoresis method on 2% agar. The amplification of the HA1 and HA2 genes were 1,100 and 1,000 bp.

Selected samples have HA1 and HA2 gene amplifiers of the right size (1,100bp and 1,000bp) according to the gene sequencing design and sending to gene sequence at the Macrogen Company in Korea.

2.5 Analysis of HA gene sequences method

The HA sequences of the H5N1 avian influenza A virus were processed and analyzed by MEGA 6.0 (Tamura *et al.*, 2013) to determine the nucleotide differentiation rate then to calculate the homology rate among the virus strains. Identification of the type A H5N1 avian influenza virus clade was performed by using the Neighbor-Joining method with 1,000 replicates of the bootstrap credibility.

3 RESULTS AND DISCUSSIONS

3.1 Results of the HA genes sequencing and comparison of genetic variation of type A H5N1

influenza virus circulating and causing disease on poultry

The 10 HA gene of type A H5N1 avian influenza virus was sequenced with the length from 1.640 to 1.695 nucleotides; result in a comparison of nucleotide and amino acid variants of virus isolates in the study was presented in Table 1 and Table 2.

The comparative results of nucleotides showed that there has been differentiation between type A H5N1 avian influenza viruses that cause disease and circulating in poultry in some provinces in the Mekong Delta in 2016. The virus strains A/Duck/TV/1605/2016 causing disease on duck in Tra Vinh had a high homology rate with A/Muscovy duck/CM/1834/2016 circulating on Muscovy ducks in Ca Mau which differentiated rate was 1.5%. Meanwhile, the virus strain A/Chick/ST/1607/2016 causing disease on chicken in Soc Trang had the highest incidence (5.5%) compared to the A/Chick/AG/0010/2016 strains circulating on chicken in An Giang. In general, the differentiation in nucleotide levels among type A H5N1 avian influenza virus strains that causes disease and circulating in poultry in the Mekong Delta provinces in 2016 was 1.5% to 5.5%. In other words, the rate of homology between type A H5N1 avian influenza strains causing disease and circulating in poultry in the Mekong Delta in 2016 was 94.5% to 98.5%. These homological rates were higher than the research of Duong Thi Thanh Thao and Ly Thi Lien Khai (2011) with the rate of homology between the virus strains isolated in Soc Trang and Ca Mau being 92-98%.

Table 1: Number of different nucleotide positions between type A H5N1 avian influenza virus causing disease and circulating in poultry in the Mekong Delta's provinces in 2016

Type A H5N1 Influenza virus causing disease on poultry	Number and percentage of nucleotides varying of avian influenza A H5N1 strains causing disease and circulating on poultry				
	Type AH5N1 influenza virus circulates on poultry				
	A/Chick/AG/0010/2016	A/Duck/CM/0057/2016	A/Duck/DT/1760/2016	A/Duck/DT/1767/2016	A/Muscovyduck/CM/1834/2016
A/Duck/TV/1605/2016	63 (3.8%)	30 (1.8%)	39 (2.4%)	64 (3.9%)	26 (1.5%)
A/Duck/CT/1606/2016	70 (4.3%)	27 (1.6%)	38 (2.3%)	64 (3.9%)	26 (1.5%)
A/Chick/ST/1607/2016	90 (5.5%)	38 (2.3%)	29 (1.7%)	60 (3.6%)	31 (1.9%)
A/Chick/CT/1613/2016	70 (4.3%)	27 (1.6%)	38 (2.3%)	64 (3.9%)	26 (1.5%)
A/Chick/CM/1635/2016	75 (4.6%)	33 (2.0%)	44 (2.7%)	69 (4.2%)	34 (2.1%)

Table 2: Number of different amino acid positions of type A H5N1 avian influenza virus that causing disease and circulating in poultry in the Mekong Delta in 2016

Type A H5N1 Influenza virus causing disease on poultry	Number and percentage of amino acids varying in type A H5N1 avian influenza strains causing disease and circulating on poultry				
	Type AH5N1 influenza virus circulates on poultry				
	A/Chick/AG/0010/2016	A/Duck/CM/0057/2016	A/Duck/DT/1760/2016	A/Duck/DT/1767/2016	A/Muscovyduck/CM/1834/2016
A/Duck/TV/1605/2016	36 (6.6%)	9 (1.6%)	4 (0.7%)	15 (2.7%)	5 (0.9%)
A/Duck/CT/1606/2016	39 (7.1%)	8 (1.5%)	5 (0.9%)	16 (2.9%)	8 (1.5%)
A/Chick/ST/1607/2016	41 (7.5%)	11 (2.0%)	4 (0.7%)	16 (2.9%)	9 (1.6%)
A/Chick/CT/1613/2016	40 (7.3%)	9 (1.6%)	6 (1.1%)	17 (3.1%)	9 (1.6%)
A/Chick/CM/1635/2016	41 (7.5%)	11 (2.0%)	8 (1.5%)	18 (3.3%)	10 (1.8%)

The different amino acids among type A H5N1 avian influenza strains causing disease and circulating in poultry in some provinces in the Mekong Delta in 2016 ranged from 0.7% to 7.5% which had a larger gap compared to nucleotide differences levels with the different rates from 1.5% to 5.5%. The strain of A/Duck/DT/1760/2016 circulating on duck in Dong Thap had the lowest differentiated rate (0.7%) compared to A/Duck/TV/1605/2016 strain causing disease on ducks in Tra Vinh and A/Chick/ST/1607/2016 causing disease on chicken in Soc Trang. Meanwhile, the strain A/Chick/AG/0010/2016 circulating on chickens in An Giang had the highest different rate (7.5%) compared to A/Chick/ST/1607/2016 causing disease on chicken in Soc Trang. Thus, the homological rate of amino acids of type A H5N1 avian influenza virus strains causing disease and circulating in poultry in some provinces in the Mekong Delta in 2016 from 92.5% to 99.3%.

Table 3: The amino acid sequence where linkage between HA1 and HA2 (cleavage site) of type A H5N1avian influenza that causing disease and circulating in poultry in some provinces in the Mekong Delta in 2016

Virus strains code	The amino acid sequence between HA1 and HA2 (cleavage site)				
A/Duck/TV/1605/2016	R	R	R	K	R
A/Duck/CT/1606/2016	R	R	R	K	R
A/Chick/ST/1607/2016	R	R	R	K	R
A/Chick/CT/1613/2016	R	R	R	K	R
A/Chick/CM/1635/2016	R	R	R	K	R
A/Chick/AG/0010/2016	R	R	R	K	R
A/Duck/CM/0057/2016	R	R	R	K	R
A/Duck/DT/1760/2016	R	R	R	K	R
A/Duck/DT/1767/2016	R	R	R	K	R
A/Muscovy_duck/CM/1834/2016	R	R	R	K	R

The result showed that the amino acid sequences between HA1 and HA2 (cleavage site) of type A H5N1 avian influenza strains causing disease and circulating in poultry in some provinces in the Mekong Delta in 2016 were similar (RRRKR), which were similar to the highly pathogenic type A H5N1 avian influenza strain 2.3.2 in the world (http://www.offlu.net/index.php?id=78) and similar to the results of the study on characteristic analysis of HA (H5) gene and NA (N1) gene of type A H5N1 avian influenza clade 2.3.2.1 strains collected in Vietnam in 2014 (Le Thanh Hoa *et al*., 2015). Thus, the result of the amino acid sequence between HA1 and HA2 was RRRKR. It can be confirmed that strains of type A H5N1 avian influenza viruses that causing disease and circulating in poultry in the Mekong Delta provinces in 2016 were a highly pathogenic strain.

3.2 Results of clade identification of type A H5N1 avian influenza causing disease and circulating on poultry in some provinces in the Mekong Delta in 2016

For clade identification of type A H5N1 avian influenza virus, MEGA 6.0 software was used to analyze and construct phylogenetic tree. Beside the viral strains sequenced in this study, the sequences of the reference strains include A/Hubei/1/2010/H5N1 (clade 2.3.2.1a; CY098758), A/barn Swallow/Hong Kong/1161/2010/H5N1 (clade 2.3.2.1b; KC357320), A/Vietnam/1203/2004 (clade 1; HM006759), A/Goose/Guangdong/1/1996 (clade 0; AF144305), A/chicken/Korea/IC546/2011 (clade 2.3.2.1c; JN807978), A/Chicken/Vietnam/NCVD-44/2007/H5N1 (CY030531), A/Chicken/Vietnam/NCVD-A937/2011/H5N1 (Clade 1.1.2; KP097925), A/Chicken/Vietnam/NCVD-swab15/2008/H5N1 (Clade 7.2; FJ842477), A/Duck/Cambodia/PV027D1/2010/H5N1 (Clade 1.1.2; JN588821), A/Hong Kong/6841/2010/H5N1 (clade 2.3.2.1c; HQ636461) were used to construct phylogenetic tree that identified the clade virus. The results were shown in Figure 1.

Analysis of the HA gene sequences and construction phylogenetic tree plotting of type A H5N1 avian influenza virus strains using MEGA 6.0 software (Tamura *et al*., 2013) identified all sequenced virus strains belonging to clade virus 2.3.2.1c.

Type A H5N1 influenza viruses circulating and causing disease on poultry were divided into two groups and the same subdivided of the A/Hong Kong/6841/2010/H5N1 (clade 2.3.2.1c) and A/chicken/Korea/ IC546/2011 (clade 2.3.2.1c). These confirmed that those strains were also belong to clade 2.3.2.1c. This result was similar to the previous study of Tien Ngoc Tien *et al.* (2016) in type A H5N1 avian influenza strains circulating in some provinces in the Mekong Delta in 2015 also belong to clade 2.3.2.1c. In addition, it can be shown that the A/Chick/AG/0010/2016 virus strains circulating on chickens in An Giang but present in the same subgroup of the causing disease type A H5N1 avian influenza virus and vice versa A/Chick/ST/1607/2016 causing disease on chickens in Soc Trang that were found in the same subgroup with viruses circulating on poultry. These results indicated that type A H5N1 avian influenza viruses circulating and causing disease on poultry had similar genetic and virulence characteristics.

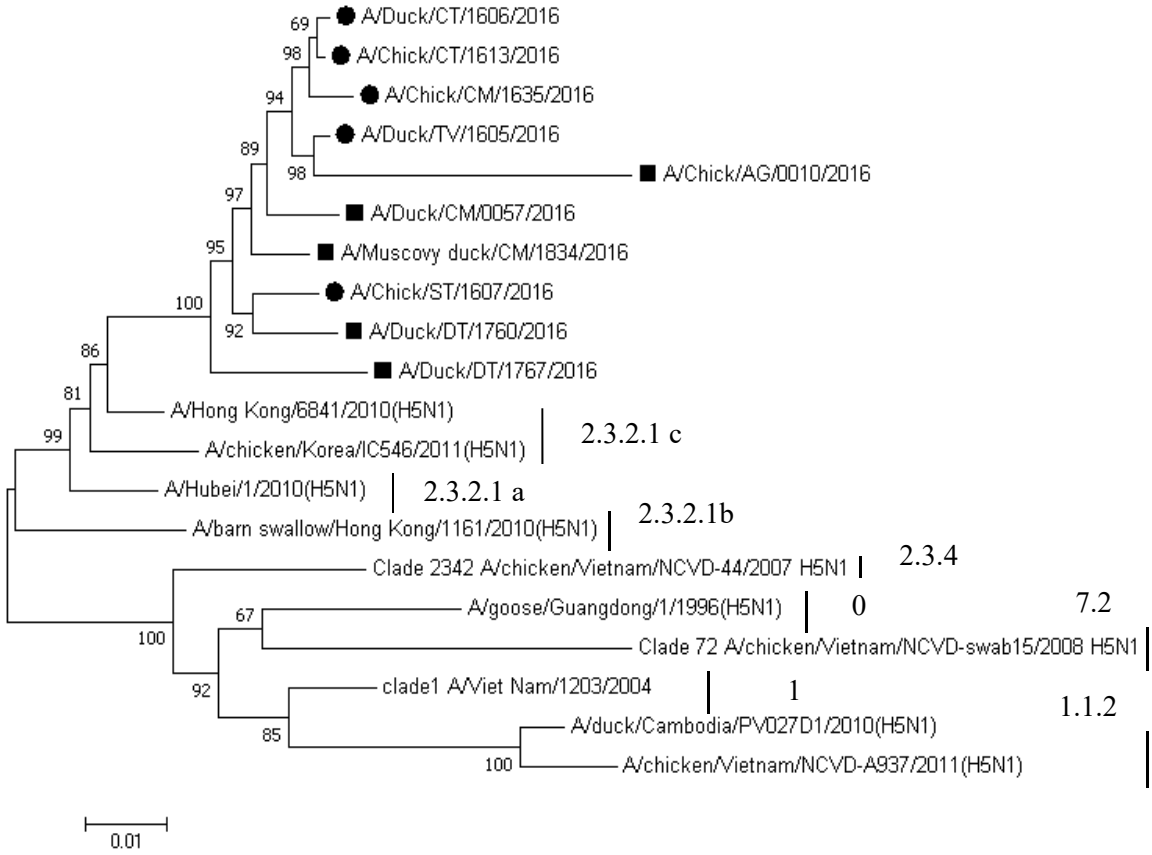

Fig. 1: phylogenetic tree of type A H5N1 avian influenza viruses

(The phylogenetic tree was built using the MEGA 6.0 software (Tamura et al., 2013) with the Neighbor-Joining method and the 1,000 repeats in Bootstap reliability coefficient. Nomenclature of virus subtypes based on WHO/OIE/FAO criteria (2014). The strains of this study were marked by black circles and squares; viruses that were marked with a black circle were the virus that causing disease in poultry; viruses marked with black squares were viruses circulating in birds)

4 CONCLUSIONS

Type A H5N1 avian influenza viruses circulating and causing disease on poultry in some provinces in the Mekong Delta in 2016 had homological rate in nucleotides from 94.5% to 98.5% and in amino acids from 92.5% to 99.3%. The sequence of amino acids at the linkage between the HA1 and HA2 virulent segments was RRRKR similar to the highly pathogenic type A H5N1 avian influenza as A/H5N1/IC546/2011 (H5N1) and A/Hubei/2010 (H5N1). Type A H5N1 influenza viruses isolated in this study are highly pathogenic strains. Types A H5N1 influenza viruses circulating and causing disease on poultry in some Mekong Delta provinces belong to clade virus 2.3.2.1c.

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