SHORT COMMUNICATION

Genotypic and Sub-genotypic Diversity of Avian Paramyxoviruses 2, 4 and 6

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A B S T R A C T

Avian paramyxoviruses (APMVs) are contagious viruses infecting multiple avian species around the globe. Subsequent to evolution, the emergence of new strains is considered to cause outbreaks worldwide. Using standard classification criteria, genotypic and sub-genotypic distribution of strains within APMV-1 is much elucidated across the globe. Nevertheless, despite a growing number of genome sequencing data for APMVs excluding those that are prototypes, there is an absolute paucity of an updated unified phylogenetic classification scheme for APMV-2, 4, and 6. Utilizing a well-recognized genetic marker (complete fusion gene), genotyping and sub-genotyping of under-studied of APMVs strains was proposed by the implementation of different reliable tools and criteria. The analysis categorized the strains of each APMV-2 and 6 into two distinct genotypes (I and II), whereas APMV-4 strains categorized into three genotypes (I, II, and III). Additionally, a total of four sub-genotypes within APMV-6 (I.1, I.2, II.1, and II.2) and five sub-genotypes (I.1, I.2, II.1, II.2, and II.3) within APMV-4 were also proposed in the current study. Though it may require a revision and update in the future with the abundance of genome sequences and subsequent analysis from wide geography, herein the outcomes are relevant to better elucidate the classification and molecular epidemiology of study-included APMVs to depict an evolution and epidemiological link among outbreaks caused by field circulatory strains.

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INTRODUCTION

Avian paramyxoviruses (APMVs), formerly known as *Avian avulaviruses* (AAvVs), emerged as a major threat to the poultry industry across the globe. The APMV-infections are endemic in various developing countries, including Pakistan, while the disease-free regions are prone to accidental outbreaks (Alexander, 2003). Wild birds including migratory and waterfowls are considered as reservoirs of APMVs and can easily transmit viruses to domestic and/or commercial poultry (Aziz-ul-Rahman *et al*., 2018, 2019). These viruses are always being of great economical concern for poultry industries and have caused devastating outbreaks (Alexander, 2003; Gogoi *et al*., 2017). The APMVs infect a wide variety of avian species but chickens and pigeons were considered as incidental hosts (Warke *et al*., 2008). The APMVs have been reported from a wide range of

countries, including African, North American, European and Asian countries (Alexander, 2003; Gogoi *et al*., 2017). Infection of APMV-1 shows clinical representation involving the nervous and digestive systems with a high mortality rate, whereas infection of APMV-2, 4, and 6 shows decrease yield and hatchability, mild interstitial pneumonia, catarrhal tracheitis, and drop in egg production (Alexander, 2003; Warke *et al*., 2008).

Avian paramyxoviruses are recently classified into three genera *Orthoavulavirus*, *Metaavulavirus*, and *Paraavulavirus* under the subfamily *Avulavirinae* of the *Paramyxoviridae* family (Kuhn *et al*., 2020). Its genome is comprised of a single negative sense polymorphic RNA that encodes six structural proteins in an order of 3′-*NP-P/V/W-M-F-HN-L*-5′ with an additional SH gene only in species-type 6 (Alexander, 2003). Owing to genomic evolution, the emergence and/or re-emergence of a novel APMVs implies that distinct strains are simultaneously

evolving and 21 species-types are now circulating worldwide (Rahman *et al*., 2018). Therefore, it ascertains a genome-based phylogenetic uniform taxonomic classification for each of the species-type to better elucidate the evolution and an epidemiological link for outbreak occurring either simultaneously or at an interval in a wide geography. In this regards, genome-based phylogenetic classification and molecular epidemiology of APMV-1 strains representing different hosts and geography are well-elucidated (Diel *et al*., 2012; Dimitrov *et al*., 2019). However, excluding those APMVs that are prototype or are limited to just a sequence, the same is not true for other APMVs that are reported from multiple hosts and different geographical areas. Therefore, a unified classification scheme is now requisite for those APMVs having enough strains as follows a well-known classification system of APMV-1 strains (Dimitrov *et al*., 2019). That being said, at the level of genotypes and sub-genotypes, we conducted a genome-based classification of APMV-2, 4, and 6 strains to investigate the molecular epidemiology of newly emerging APMV strains. Moreover, the current study will provide a foundation to better comprehend the evolution and molecular epidemiology of closely-related APMVs reported from different hosts and locations worldwide.

MATERIALS AND METHODS

Other than APMV-1, there is a paucity of genome sequencing data for all known species-types. Among these, specie-types 3, 5, 7, 10-21 are those that have only a genome sequence in a public database (http://www.ncbi.nlm.nih.gov/). Considering previously described criteria about three independent strains for classification of taxa representing specific geography within a specific period for designing a genotype and/or sub-genotype (Dimitrov *et al*., 2019), sequences of species-type 8 and 9 were also excluded. By September 2020, the public database (http://www.ncbi.nlm.nih.gov/) had a total of 280 nucleotide sequences that were comprised of 47 sequences of APMV-2 (complete genome = 8, complete F gene = 4, partial L gene = 1 and complete HN gene = 34), 171 sequences of APMV-4 (complete genome = 12, complete F gene = 77, partial F gene = 78, partial P gene = 1, partial L gene = 1 and complete HN gene = 2) and 62 sequences of APMV-6 (complete genome = 11, complete F gene = 14, partial F gene = 14, partial L gene = 13, partial P gene = 4, complete M gene = 3, partial P gene = 2, and complete HN gene = 1). Although, four different classification criteria are being used for the required purpose (Diel et al., 2012; Dimitrov et al., 2019); however, since the database did not have all of that relevant genome sequence information, therefore, we were limited to use only complete F gene sequences for essential analysis. With this limitation, the data analysis included a total of 126 complete F gene sequences retrieved either directly (n = 95) or derived from the complete genome sequences (n = 31).

All complete *F* gene sequences were labeled according to their species-type and aligned by ClustalW methods in BioEdit® version 5.0.6 (Hall, 1999). To annotate the topology of a phylogenetic tree, a Maximum Likelihood (ML) statistical method of the Tamura-Nei model with 1000 bootstraps was applied in the MEGA® version 6.0 and the goodness-of-fit of the model was measured using the Akaike Information Criterion (Tamura *et al*., 2013). For the classification of different genotypes and sub-genotype as per nucleotide divergence cut-off (Dimitrov *et al*., 2019), a pairwise sequence comparative analysis was performed in the MEGA® version 6.0. To further confirm the reliability of the comparative genomic analysis, a pairwise nucleotide identity and/or divergence matrix was also generated using Sequence Demarcation Tool (SDT) version 1.2 in muscle mode (Muhire *et al*., 2014). The sequence identity cut-off value was adjusted at 90% to demarcate strains representing different species-types of APMVs. For the subject matter, all 126 sequences were submitted to the DIVEIN web tool (available at https://indra.mullins.microbiol.washington.edu/DIVEIN/) according to each species-type. Based upon individual genetic divergence among different strains of under-studied APMVs, a histogram representing the genetic distance/divergence was also computed.

RESULTS AND DISCUSSION

Utilizing a well-recognized genetic marker (complete *F* gene), we retrieved the so far reported APMV species-type 2, 4, and 6 genome sequences from National Centre for Biotechnology Information (NCBI) and classified at the level of genotypes and sub-genotypes by the implementation of different reliable tools. The International Committee on Taxonomy of Viruses (ICTV) *Paramyxiviridae* study group recommends at least a complete coding sequence/designated evolutionary marker for classification of APMVs strains into different taxonomic nodes using pre-determined cut-off values (Diel *et al*., 2012; Dimitrov *et al*., 2019). Among the different genetic markers, the complete *F* gene is capable of generating evolutionary and phylogenetic information (Diel *et al*., 2012; Dimitrov *et al*., 2019). Therefore, based upon complete *F* gene sequences, sequence identity analysis revealed a varying nucleotide divergence for distribution of genotypes (6-18%) and sub-genotypes (5-8%) among the under-studied strains representing APMV-2, 4, and 6. This is important because different cut-off values are being used to classify a virus at each level of a taxonomic node. For instance, using complete genomes or complete *F* or *L* gene sequences, APMV-1 strains with a nucleotide divergence ≥10% and ≥5% is considered enough for a novel genotype and sub-genotype, respectively (Dimitrov *et al*., 2019). Although the genomic- and residue-based diversity for all sub-species (species-type 1-21) within *Avulavirinae* has recently been reported based on the complete genome and/or gene sequences (Rahman *et al*., 2018), however, it was limited to only a representative strain of each species-type and, despite an increasing genome database, a comprehensive genomic analysis of different variants within APMV-2, 4 and 6 remained elusive. Hence, following a well-established genetic marker (complete *F* gene), the current study provides a uniform classification of these APMVs at the level of genotypes and sub-genotypes which can be used to elucidate the molecular epidemiology of these APMVs worldwide.
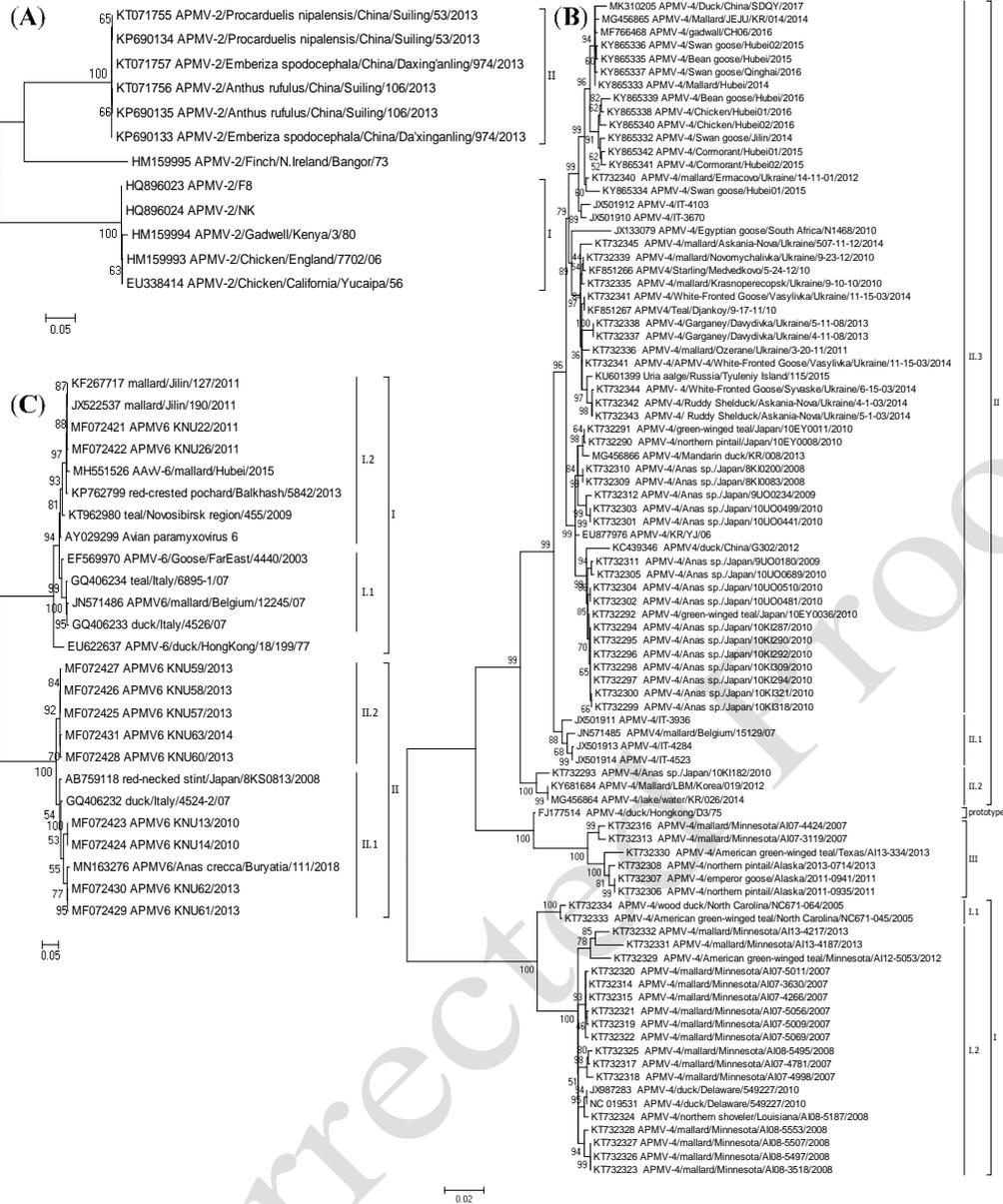
Fig. 1. Phylogenetic analysis of complete *F* gene sequences of so far reported strains of APMV-2 (A), APMV-4 (B), and APMV-6 (C). The Maximum Likelihood (ML) statistical method of the Tamura-Nei model with 1000 bootstraps was used for the analysis of the evolutionary relationship between strains using MEGA® 6 software.
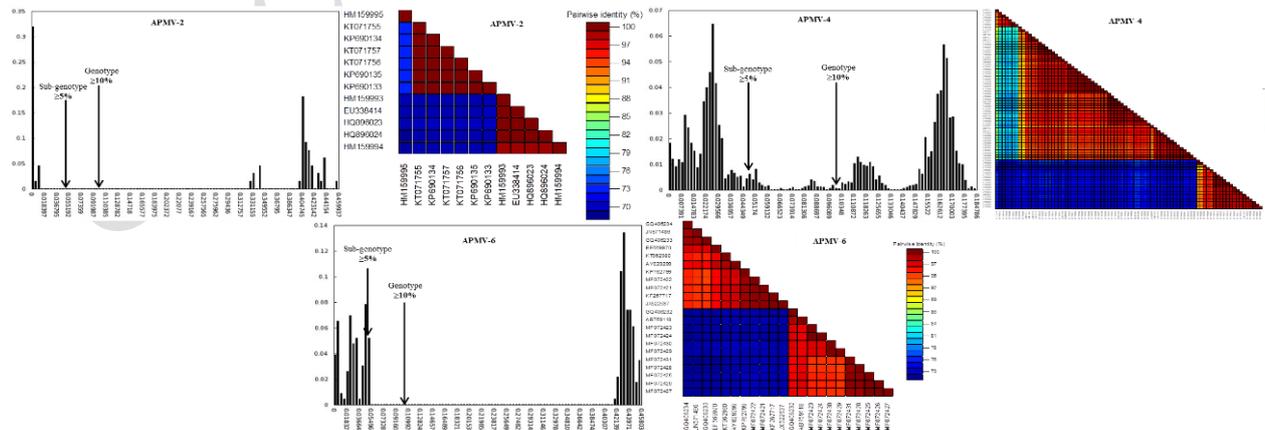

Fig. 2. The nucleotide divergence of the study-included APMV-2, 4, and 6 strains. The genetic distance (X-axis) based histogram presenting frequency distribution (Y-axis) of pairwise distance among strains with cut-off values of genotype (10%) and sub-genotype (5%). In a color-coded pairwise identity matrix, each colored cell represents a percentage identity score between two sequences (one indicated horizontally to the left and the other vertically at the bottom).

Together with the outcomes of sequence similarity analysis, the phylogenetic analysis identified two genotypes (I and II) within APMV-2. The genotype I comprised of strains isolated from Euro-African regions during 1956-2006 whereas, genotype II included strains reported from China in 2013 (Fig. 1A). Viruses within APMV-4 were classified into three distinct genotypes (I, II, and III). Viruses within genotype I and II were further divided into two (I.1 and I.2) and three sub-genotype (II.1, II.2, and II.3), respectively (Fig. 1B). Genotype I and III included strains that were reported from the USA during 2005-2013 whereas genotype II had strains reported from European and Asian countries during 2006-16 (Fig. 1B). Viruses of APMV-6 were clustered into two distinct genotypes (I and II) and these genotypes I and II were further classified at the sub-genotypes level as I.1 and I.2 and, II.1 and II.2, respectively. Sub-genotype I.1 and I.2 comprised of viruses reported from Italy, American and Asian countries during 2003-09 and 2011-15, respectively. While sub-genotype II.1 and II.2 had strains reported from Italy and Japan during 2007-13 and 2013-14 exclusively from Japan (Fig. 1C). Based on the geographical distribution pattern, the strains within APMV-4 have previously been classified (Choi *et al*., 2013; Tseren-Ochir *et al*., 2017). Tseren-Ochir *et al*. (2017) designated viruses of APMV-4 into two clades as an old-world/Eastern hemisphere clade comprising of Euro-Asiatic and African strains, and new world/Western hemisphere clade comprising of American strains. Though it may provide some preliminary assessment for prevailing strains, such a geographic-pattern-based classification may raise controversies and, therefore, may not be considered reliable as per known criteria for a uniform classification into genotypes and sub-genotypes (Dimitrov *et al*., 2019). Indeed, with a substantial increase in the number of genome sequences in the future, following uniform classification criteria such as presented in this study is necessary for a more precise classification at the genotypes and sub-genotypes levels. Taken together, such a genetic diversity within a specific APMVs highlight their potential to evolve, resulting in either emergence and/or re-emergence of novel genotypes and sub-genotypes across the globe (Rahman *et al*., 2018; Dimitrov *et al*., 2019).

Exploring nucleotide divergence among study-included sequence database for classification at level of genotypes and sub-genotypes, we found an agreement between the outcomes of analysis using different evolutionary tools such as phylogeny analysis, sequence similarity analysis, sequence demarcation tool, and pairwise distance calculation histogram (Fig. 2). The histogram displayed a multimodal curve indicating genetic distance among strains of APMV-2, 4, and 6. It showed a higher genetic distance for strains within APMV-2 (n = 12, up to 0.459, 25.27±0.024) followed by APMV-6 (n = 25, up to 0.458, 24.31±0.013) and APMV-4 (n=89, up to 0.198, 8.74±0.001). Similarly, the sequence demarcation tool revealed a variable range of nucleotide divergence among strains of each species-type. It was found to be 18.72-20.19% for APMV-2, 11.16-15.29% for APMV-4 and 25.41-27.90% for APMV-6 (Fig. 2). These

values of nucleotide identity/divergence of all targeted APMV strains are consistent with well-established classification (Dimitrov *et al*., 2019). Summarizing together, the current study revealed that APMV-2, 4, and 6 strains could be classified at genotypes and sub-genotype levels using different reliable tools including pairwise distance calculation and sequence demarcation. Using pre-determined criteria with the help of these tools, the current study proposed a unified classification of APMV-2, 4, and 6 at genotypes and sub-genotypes level. Therefore, the current study concluded that such an established classification is expected to better elucidate the molecular epidemiology, evolutionary relationship, and emergence and/or re-emergence of novel strains of APMVs.

Authors contribution: AR apprehended the idea; AR, MABS, AM, and MZS did the analysis and compiled the whole manuscript.

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