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Emergence of XIII.2.2 genotype of Avian Avulavirus-1 with unique FPCS site in India

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ABSTRACT

In the present study, two isolates namely D162 and D165 obtained from Newcastle disease outbreaks in backyard poultry in Tamil Nadu were subjected to phylogenetic analysis using complete Fusion (F) gene sequence to understand the circulation and evolution of Avian Avulavius-1 (AAv1). Further, these isolates were pathotyped based on mean death time and intra-cerebral pathogenicity index. Pathotyping and genotyping of D162 and D165 showed that these isolates belonged to velogenic pathotype and XIII.2.2 sub-genotype. In addition to this, the Fusion Protein Cleavage Site (FPCS) of F gene was analysed for amino acid sequence pattern. The amino acid sequence of FPCS of both D162 and D165 was ^{112}RRRKRF^{117}, which is unique among XIII.2.2 genotypes of AAv1, whereas it is common among Pigeon Paramyxovirus. It was hypothesized that cross-species infection by AAv1 has led to emergence of isolates with increased virulence.

Keywords: Avian Avulavirus-1, Genotyping, Newcastle disease virus, Pathotyping, Phylogenetic tree, Viscerotropic velogenic

Poultry farming plays an important role in Indian economy and has metamorphosed from backyard avocation to a strong agro-based industry, where infectious diseases, especially Newcastle Disease (ND) continue to remain a major impediment (Doyle 1927). It is caused by a single stranded negative sense RNA virus namely Avian Avulavirus-1 (AAv1), formerly known as Newcastle disease virus. AAv1 is grouped under *Avulavirus* genus under Mononegavirales order of Paramyxoviridae family (King *et al.* 2012). AAv1 is reported worldwide and the various strains of AAv1 can be categorized by pathotyping and genotyping.

Based on the clinical signs and lesions produced in chickens, AAv1 can be classified into five pathotypes namely viscerotropic velogenic (VVND), neurotropic velogenic (VNND), mesogenic, lentogenic and avirulent (OIE 2012). In addition to this, the velogenic, mesogenic and lentogenic strains can also be differentiated based on mean death time (MDT) in embryonated chicken eggs and intra-cerebral pathogenicity index (ICPI) in day-old chicks. Although AAv1 isolates can be pathotyped based on MDT and ICPI, genetic diversity within and among the pathotypes has been reported (Diel *et al.* 2012).

Genotyping of AAv1 isolates using phylogenetic analysis of complete sequence of F gene resulted in clear demarcation of genotypes and hence is regarded as a widely accepted method of classification (Diel *et al.* 2012). Recently, an updated genotype classification with revised nomenclature was reported (Dimitrov *et al.* 2019). According to this system of classification, Class I AAv1 consists of genotype 1 with three sub-genotypes, whereas Class II AAv1 encompasses 20 genotypes from I to XXI excluding XV. With occasional emergence of new isolates during outbreaks across India, it is necessary to genotype the isolates in order to understand the circulation and evolution of AAv1 (Dimitrov *et al.* 2019). Therefore, in the present study, two isolates namely D162 and D165 obtained from outbreaks in backyard poultry in Tamil Nadu were subjected to phylogenetic analysis using complete F gene sequence. In addition to this, the Fusion Protein Cleavage Site (FPCS) of F gene was analysed for amino acid sequence variation. Further, these isolates were pathotyped based on MDT and ICPI in order to corroborate the same with genotype classification.

MATERIALS AND METHODS

Virus: Two AAv1 field isolates namely D162 and D165 obtained from native village chicken during Newcastle disease outbreak in South India were used in this study. These isolates were propagated in allantoic cavity of 9-day old specific pathogen free (SPF) embryonated chicken egg (ECE) and the amnio-allantoic fluid (AAF) was harvested.

Extraction of total RNA and reverse transcription:

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Total RNA was extracted from D162 and D165 AAF using Trizol (Life Technologies, USA, Cat # 15596-026) as per manufacturer's instructions. Reverse transcription was done with 1 µg of total RNA using Thermoscript cDNA synthesis kit (Cat # 12236-014, Invitrogen, USA) following manufacturer's instructions.

Primers: The forward primer 5'-ATG GGC CCC AGA CCT TCT ACC AA-3' and reverse primer 5'- ATG TGA CTC TGG TAG GAT AAT C-3' were used to amplify the complete F gene of the D162 and D165. The primers were designed and validated using Primer3 (Untergasser *et al.* 2012) and Oligoanalyzer software v2.1. respectively.

PCR amplification and purification: The complete F gene of D162 and D165 was amplified using Phusion high fidelity PCR kit (Cat# E0553S, NEB, USA) as per manufacturer's instructions. In brief, the reaction mixture comprised of 50 µl of 5× Phusion HF buffer, 0.2 mM of dNTPs, 0.5 µM of each primer, 3% of DMSO, 1 unit of Phusion DNA polymerase and 10 ng of cDNA template in a final reaction volume of 50 µl. The reaction cycle involved initial denaturation at 98°C /30 s followed by 35 cycles of denaturation at 98°C /10 s, annealing at 57°C /10 s and extension at 72°C /30 s /kb with a final extension at 72°C /5 min. The amplified PCR product of D162 and D165 were gel purified using Nucleospin Gel and PCR cleanup kit (Cat#740609.50, Machery-Nagel, Germany) and was subjected to nucleotide sequencing.

Nucleotide sequencing and phylogenetic tree construction: The complete F gene of D162 and D165 were aligned and subjected to ClustalW multiple sequence alignment along with 137 sequences of pilot dataset of AAv1 genotypes (Dimitrov *et al.* 2019) and phylogenetic tree was constructed using maximum-likelihood algorithm (Tamura and Nei 1993) with 1000 bootstrap replicates using Mega 7.0 software (Kumar *et al*. 2016).

Deduction of amino acid sequence in FPCS region: Upon construction of phylogenetic tree, the nucleotide position of FPCS of D162 and D165 between 336 and 351 of F gene that corresponds to amino acid sequence between 112 and 117 position was analyzed.

Biological characterization of D162 and D165: MDT and ICPI were carried out in 9-day old SPF-ECE and day-old SPF chicks respectively as per international standards (OIE 2004), and approved by Institutional Animal Ethical Committee (IAEC) (3028/DFBS/B/2014).

RESULTS AND DISCUSSION

ND was first reported in India in 1930 (Kylasam 1930), and ever since then, it has been enzootic in India with regular outbreaks. AAv1, the causative of ND, is a single serotype with five pathotypes that vary in virulence to chickens. VVND and VNND are virulent strains of AAv1 that cause viscerotropic and neurotropic lesions, respectively, leading to death. Mesogenic strains are moderately virulent strains which affect respiratory and nervous system. Lentogenic strains are less virulent strains that cause sub-clinical respiratory infection. AAv1 has 20 genotypes as per the updated classification (Dimitrov *et al.* 2019). Genotyping of AAv1 isolates was done based on their binding to monoclonal antibodies (Alexander *et al.* 1987), restriction fragment length polymorphism of partial coding sequence of Fusion (F) protein gene (Ballagi-Pordany *et al.* 1996), differences in the nucleotide or amino acid variations of F gene (Aldous *et al.* 2003) and phylogenetic analysis using complete sequence of F gene (Diel *et al.* 2012). The genotype classification based on F gene sequence has gained importance as it helps in elucidating the evolution and circulation pattern of AAv1. It has also been suggested that vaccines prepared from same genotypes offer better protection in chickens affected with virulent viruses belonging to same genotype (Hu *et al.* 2009, Miller *et al.* 2009). Hence, identification of genotypes of AAv1 circulating in India is necessary.

In the present study, two isolates namely D162 and D165 were subjected to biological and molecular characterization to identify the pathotype and genotype. Pathotyping of AAv1 by biological methods involves estimation of MDT and ICPI. MDT of velogenic, mesogenic and lentogenic strains is <60 h, 60 – 90 h and >90 h, whereas ICPI is 1.8 to 2.0, 0.8 to 1.5 and <0.7, respectively (Allan *et al.* 1978). In this study, MDT of both D162 and D165 was 48 h, whereas ICPI of D162 and D165 was 1.8 and 1.77 respectively. On the basis of MDT and ICPI, it was confirmed that both D162 and D165 were velogenic AAv1. In a previous study, it was reported that backyard chicken infected with a velogenic AAv1 with ICPI of 1.59 presented no clinical signs (Ananth *et al.* 2008). However, D162 and D165 caused high mortality in backyard poultry. This variation in pathogenicity in backyard poultry could be due to higher ICPI value (Tirumurugaan *et al.* 2011, Dey *et al.* 2014). The amino acid sequence at FPCS region varies between lentogenic and mesogenic/velogenic strains, which aids in pathotyping of AAv1 isolates by molecular method (Collins *et al*. 1994). The lentogenic strains possess ^{112}G/E-K/R-Q-G/E-R-L^{117} with few basic amino acids, whereas mesogenic/velogenic strains possess ^{112}R/K-R-Q-R/K-R-F^{117} with multiple basic amino acids (Nagai *et al*. 1976, Glickmann *et al*. 1988). In the present study, amino acid deduction of FPCS region of both D162 and D165 showed the pattern ^{112}RRRKRF^{117}. The deduced amino acid sequences matched with other standard virulent strains like Hertz/33 and Texas GB. These two isolates were found to have the amino acid phenylalanine (F) at residue 117, which is a characteristic feature of virulent strains of AAv1. It has been already established that a pair of basic amino acid lysine (L) or arginine (R) at 115 and 116 respectively along with phenylalanine at 117 and basic amino acid arginine at 113 determines the virulence of AAv1 (OIE 2012). D162 and D165 were found to have basic amino acids at positions 113, 115 and 116 with phenylalanine at position 117, which confirmed that both the isolates belonged to velogenic pathotype.

Genotyping of AAv1 isolates by phylogenetic construction using complete F gene sequence is reported

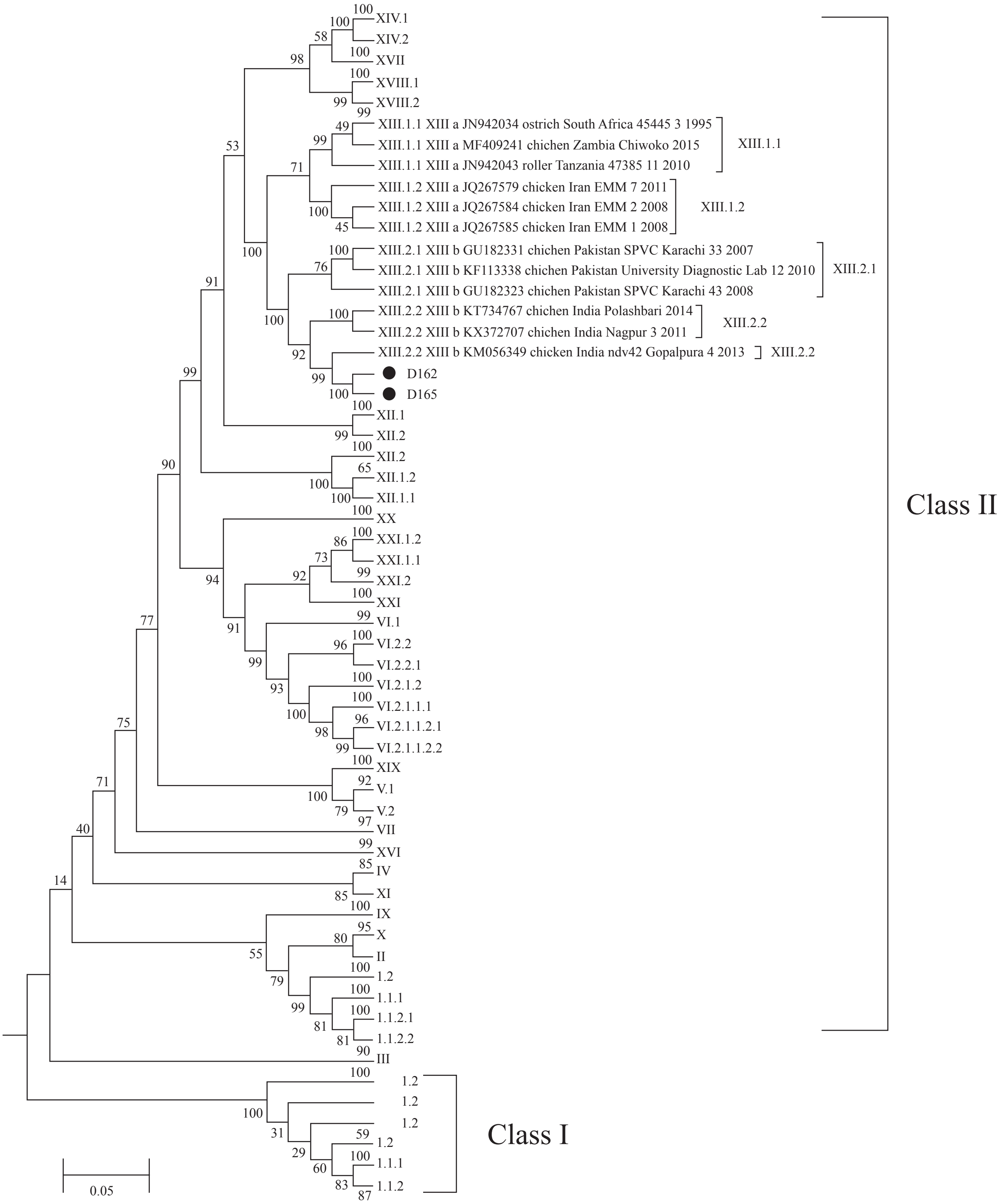

Fig.1. Phylogenetic tree of AAv1 isolates using complete F gene sequence.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The tree with the highest log likelihood (-27250.47) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pair-wise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 139 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1364 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

to be significant to identify the genotype. Ever since the first report of ND in 1926 (Kraneveld 1926), several genetic groups have been reported among AAv1 (Aldous *et al.* 2003) and phylogenetic studies have shown that viruses of distinct genotypes undergo simultaneous evolutionary changes in different geographical locations of the world (Miller *et al.* 2010). This evolutionary variation has been reported to complicate the disease control and diagnosis (Cattoli *et al.* 2010), suggesting use of genotype homologous vaccine over genotype heterologous vaccine (Miller *et al.* 2009). Further, there are no diagnostic tests available to detect new genetic variants of AAv1 (Diel *et al.* 2011), suggesting the importance of molecular surveillance of AAv1. In the present study, phylogenetic analysis of complete F gene sequence of D162 (Accession Number: KX242342) and D165 (Accession Number: KX710211) along with 137 sequences of Class I and Class II AAv1 genotypes and sub-genotypes, positioned both these isolates under genotype XIII.2.2 (Fig.1). This is an emerging genotype that has already been reported in western part of India (Jakhesara *et al.* 2016) and Pakistan (Shabbir *et al.* 2013). Further, when this study was ongoing, FPCS region of isolates classified under genotype XIII reported either ^{112}RRQRRF^{117} or ^{112}RRQKRF^{117} amino acid sequence pattern. However, D162 and D165 showed a unique amino acid pattern ^{112}RRRKRF^{117}. This unique pattern was then reported only in one isolate of genotype XIII reported from Pakistan (Shabbir *et al.* 2013) and none of the isolates in XIII genotype in India were found to possess this pattern. Moreover, this amino acid pattern in FPCS region was observed in many pigeon paramyxovirus (PPMV-1) isolates (Kwon *et al.* 2003). Hence, it was hypothesized that these two isolates D162 and D165 could have re-emerged consequent to cross-species infection among birds.

In conclusion, pathotyping and genotyping of D162 and D165 isolates confirmed that these isolates belong to velogenic pathotype and sub-genotype XIII. The FPCS sequence of these isolates showed an amino acid pattern ^{112}RRRKRF^{117}, normally observed in PPMV-1 isolates. However, PPMV do not produce severe lesions in chicken and have been classified as mesogenic previously. On perusal of literature, it has been observed that AAv1 isolates from pigeon with ^{112}RRRKRF^{117} FPCS pattern were virulent in chickens (Kwon *et al.* 2003). However, pathogenicity of pigeon isolates with ^{112}RRRKRF^{117} at FPCS needs a thorough study before concluding that AAv1 gains virulence in pigeons and that new genotypes of AAv1 in chickens could emerge consequent to cross-over of virus among bird species.

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