



## Differential detection and molecular characterization of multiple avian oncogenic viruses in backyard poultry of Tamil Nadu, India

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### ABSTRACT

The avian oncogenic viruses include Mareks disease virus (MDV), Avian Leukosis virus (ALV) and Reticuloendotheliosis virus (REV). All the three viruses as etiological agents of single and/or multiple oncogenic infections were reported previously in broiler and layer chickens. The present study describes the detection of these multiple oncogenic viruses by multiplex PCR from suspected samples collected from the native chicken of Tamil Nadu, a southern state of India. Out of 51 samples tested, 10 were found positive for MDV and 25 positive for ALV subgroup E and 6 samples positive for REV. Further, the representative samples were subjected to sequencing and phylogenetic analysis. The molecular characterisation of the present MDV strains showed the close proximity with strains from Tamil Nadu, Punjab, and Kerala states of India with bootstrap value of 97%. Further, the current REV strain showed close relatedness with the previously known Tamil Nadu REV isolate with bootstrap value of 66%. The ALV strain identified from the backyard poultry was found to be endogenous virus and showed close identity with ALV-E China isolate with bootstrap value of 78%.

**Keywords:** Mareks disease, Multiplex PCR, Native chicken, Oncogenic viruses, Phylogenetic analyses, Reticuloendotheliosis

Mareks disease is one of the commercially significant, worldwide prevalent major oncogenic disease of poultry, resulting in T-cell lymphomas and tumors of many visceral organs, neurological disorders and immunosuppression, hence leading either directly to mortality or health problems among the affected chickens (Sun *et al.* 2017). It is caused by Gallid alphaherpesvirus-2 (Serotype 1/MDV 1) of the genus *Mardivirus* and is grouped together with relative strains serotype 2/MDV 2 and Meleagrid alphaherpesvirus serotype 3 / MDV 3, under the family Herpesviridae (Adams *et al.* 2016). The other oncogenic poultry viruses include avian leukosis virus (ALV), an alpha retrovirus and reticuloendotheliosis virus (REV), a gamma retrovirus. ALV are further grouped into avian subgroup viruses A to J mainly based on the host range, viral envelope interference, and pattern of cross-neutralization (Payne 1991). Among these ALV subgroup A and subgroup B causes lymphoid leukosis in age old layer birds, whereas ALV-J causes myeloblastosis in broiler chickens (Fadly and Venugopal 2008) and ALV-E is an endogenous virus that usually is detected as contaminant in viral vaccines (Zavala and Cheng 2006). The REV is also a neoplastic virus causing

reticuloendotheliosis characterised both by bursal and T-cell lymphomas and further results in immunosuppression (Witter and Fadly 2003, Sun *et al.* 2017).

These days the Mareks disease, which is much more prevalent in young chicken is also found commonly in layer birds and similarly avian leukosis virus as ALV-J is common in broiler chicken too. Hence phenomenon of age prevalence among the avian oncogenic diseases is not uniform. Multiple oncogenic viral infections also are not uncommon and were reported from many countries such as China (Qin *et al.* 2010), Israel (Davidson and Borenstein 1999, Davidson 2009) and India (Gopal *et al.* 2012). Further, ALV and REV were detected as vaccine contaminants in commercial vaccines for Mareks disease (Fadly and Witter 1997). The macroscopic lesions caused by these three independent viruses often are indistinguishable, overlapping and not conclusive of any single type of infection. Hence, the multiplex PCR approach could be the presumptive diagnostic technique for rapid and simultaneous screening of these three avian oncogenic viruses.

Recently, backyard poultry is gaining momentum in India due to growth of both organic and integrated farming activities. Further mapping of these avian oncogenic viruses in backyard native chicken does not have the data as much as like that of layer and broiler birds. Further, these native backyard chicken flocks were not reported to be vaccinated

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against any of these poultry oncogenic viruses and even confirmed cases are not well documented. The reports on these neoplastic diseases in the native chicken are scanty and few are made from Ethiopia (Duguma *et al.* 2005), Italy (Mescolini *et al.* 2019), Nigeria (Adedeji *et al.* 2019) and South America (Chacón *et al.* 2019). There are literature that describe the circulation of MDV in commercial poultry flocks of India (Prathibha *et al.* 2018, Puro *et al.* 2018, Raja *et al.* 2009, Suresh *et al.* 2015). Further, the pathological aspects of MD in native chickens have also been described from southern parts India (Balasubramaniam *et al.* 2017). However, the molecular detection and characterisation of these oncogenic viruses is not well documented from native chicken of Southern India. Hence, screening of multiple avian oncogenic viruses of native chicken was aimed in this study by molecular PCR and sequence analyses.

#### MATERIALS AND METHODS

**Collection of samples:** The samples were collected from Kanyakumari, Tirunelveli and Madurai districts of Tamil Nadu, one of the southern state of India. The backyard poultry birds suspected for classical Mareks disease or lymphoid leukosis and showing the visceral tumors were included in the study. Many of these birds are of Aseel breed and some are improved low input chicken varieties, viz. vanaraja, giriraja and gramapriya. A total of 51 samples were collected, of which 39 were from visceral tumours (liver, spleen, kidney, proventriculus, gizzard) of native chickens presented for post mortem examination; 8 feather follicle and 4 blood samples were collected from live suspicious birds.

**Histopathology:** The tissue samples from chicken (n=7) that showed remarkable lesions were formalin (10%) fixed for preliminary examination by standard histopathology technique after staining with hematoxylin-eosin.

**DNA isolation and PCR:** Tissue samples, blood and feather follicle samples were used for DNA extraction by using commercial DNA isolation kit (Qiagen, Germany). Multiplex PCR oligonucleotide primers utilised in this work (Table 1) were designed for simultaneous screening of these three poultry oncogenic viruses and the same PCR amplification conditions were followed as described by Gopal *et al.* (2012). Further, the ALV positive DNA

samples were analysed by subgroup specific PCR (Silva *et al.* 2007).

**Sequence analysis:** Positive PCR products have been gel purified and analysed for sequencing. The sequences obtained were further confirmed by BLASTn tool for checking homology and comparative sequence analyses were performed using published sequences of MDV and REV available in the GenBank database. Sequence identity levels were determined by using Genetool 1.0 software. A phylogenetic tree was constructed based on nucleotide sequences using the bootstrap test of phylogeny with the neighbour-joining method provided in MEGA 11.0.10 software.

#### RESULTS AND DISCUSSION

The laboratory diagnosis of avian oncogenic viruses includes histopathology and molecular techniques. In the present study, the histopathological examination of various visceral organs collected from suspected samples (n=7 birds) showed pleomorphic infiltration of lymphoid

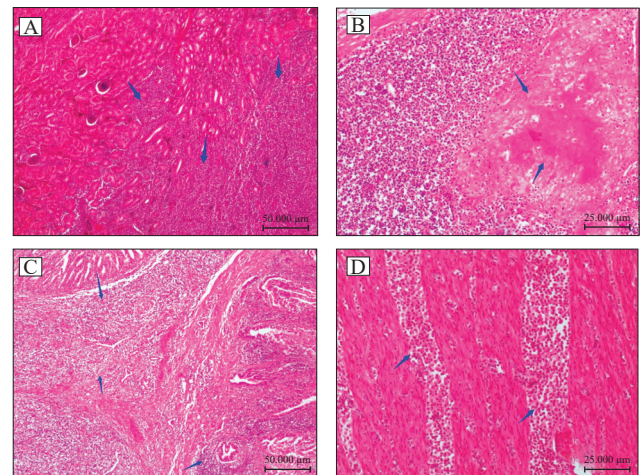


Fig. 1. Histopathology of organs suspected for viral oncogenesis: A. Kidney showing diffuse infiltration of pleomorphic lymphocytes (arrow) in the interstitium (H and E, Bar = 50 µm); B. Spleen with extensive lymphomatous infiltration and compressed narrow red pulp with an area of necrosis (arrow) (H and E, bar = 25 µm); C. Accumulation of pleomorphic lymphocytes in the submucosa (arrow) of proventriculus (H and E, bar = 50 µm); D. Infiltration of lymphocytes (arrow) in between the muscle fibers of gizzard (H and E, bar = 25 µm).

Table 1. Multiplex oligonucleotide PCR primers used in the study

Virus	Primer sequence (5'-3')	Target gene	Annealing temperature	Amplicon size	Accession number	Reference
MDV		<i>meq</i>	55°C	856 bp	EF523390.1	Gopal <i>et al.</i> 2012
F	CTGACGGCCTATCTGAGGAG					
R	GGAAACCACCAGACCGTAGA					
ALV		<i>p27</i>	55°C	613 bp	AY013303.1	
F	CCCGATCACTATGGCAGAAG					
R	GGCTATGCCTTGATCCGTAA					
REV		<i>LTR</i>	55°C	204 bp	FJ496333.1	
F	TGAGGGAAAATGTCATGCAA					
R	ATCCCTACCCACCCAGTAG					

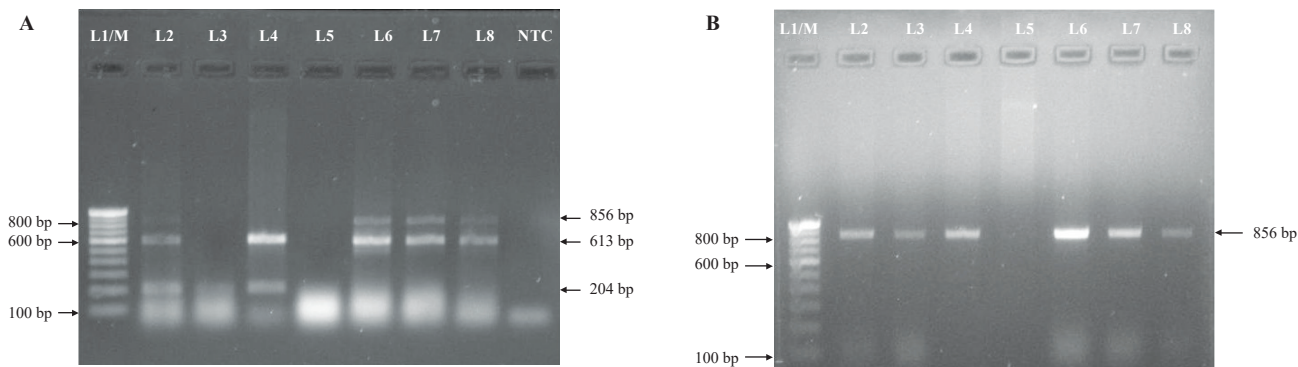


Fig. 2. A. Screening of samples by multiplex PCR. L1/M: 100 bp marker; L2: MDV, ALV and REV positive; L3: REV positive; L4: ALV and REV positive; L5: Negative sample; L6,7,8: MDV and ALV positive; L9: NTC. B. Screening of samples by MDV specific PCR. L1/M: 100 bp marker; L2-4 and 6-8: MDV positive; L5: Negative sample.

cells suggestive of Mareks disease and are shown in Fig. 1 A-D. Apart from lesions in liver (n=5) and spleen (n=4), the distinctive lesions in kidney (n=2) proventriculus and gizzard (n=2) with positive pleomorphic lymphocytic infiltration could also be seen. None of the samples showed confirmative microscopic lesions suggestive of either ALV or REV.

Though, MDV shows specific pleomorphic lymphoid infiltration (Balachandran *et al.* 2009), the histopathology of ALV and REV are not pathognomonic (Gopal *et al.* 2012). Multiple infections also cause confusion over final diagnosis. Further, the virus isolation is time consuming and method of isolation of one virus might miss the other virus in case of mixed infections. Hence, PCR is a molecular method used in the differential diagnosis of these three poultry oncogenic viruses (Davidson 2005). The results of current study by molecular detection of these avian oncogenic viruses from DNA samples of tissue, feather follicle and blood samples were shown in Table 2. The gel pictures were shown in Fig. 2 A and B. Out of 51 samples tested, 10 samples were MDV positive, 25 samples ALV positive and 6 samples were found to be REV positive. These types of multiple infectious avian oncogenic viruses' positive results by multiplex PCR were reported in other previous work done in layer farms of Southern India (Gopal *et al.* 2012).

The amplicons were sequenced and further analysed by BLASTn analysis of sequences to confirm the identity of viruses. The aligned sequences were used for phylogenetic tree construction. The results are shown in Fig. 3-5. Phylogenetic analysis of partial (799 bp) MDV *meq* sequence (GenBank accession number: MT648226) which has 100% identity with another submitted *meq* sequence MT648227 (779 bp), showed significant homology

with formerly known isolates from Tamil Nadu, Punjab, and Kerala states of India with bootstrap value of 97% (Fig. 3). In India, less effective bivalent vaccination (HVT and avirulent serotype 2 vaccines) is commonly practiced rather than serotype 1 vaccination which also worsens the condition in the MDV transmission. The incidence of MD by Indian strains was reported to be 57.5% and 25%, respectively, in birds immunized with monovalent and bivalent MDV vaccines, compared to 100% in non vaccinated birds (Suresh *et al.* 2015). Therefore, sequence identification of recent MDV field isolate is very much important and thereby the effective subunit vaccines can be employed in the immunisation programmes to contain this disease (Boodhoo *et al.* 2016).

The results of ALV subgroup specific PCR in this study, confirmed ALV subgroup E only rather than other important ALV-A, B or J viruses. All the 25 ALV positive samples from the current investigation were positive for ALV subgroup E and their representative sequence also confirmed its identity (GenBank accession number: MT648228). The ALV strain identified in the study was found to be endogenous virus in concurrence with the previous study conducted in layer flock (Gopal *et al.* 2012). The phylogenetic tree analysis revealed its close identity with ALV-E china isolate with bootstrap value of 78% (Fig. 4). Based on the surveillance report, ALV-A and B subgroups are more commonly isolated from outbreaks of lymphoid leukosis than ALV-C and D (Fadly and Venugopal 2008). Although ALV-E is endogenous virus, less important in disease diagnosis, it can participate in recombination process with exogenous virus to induce neoplasm (Smith and Fadly 1988). So, development of ALV-E free chicken lines is very important in the eradication process of avian oncogenic viruses.

The phylogenetic analysis of REV *LTR* sequence

Table 2. Screening of samples by multiplex PCR to detect oncogenic poultry viruses

Type of sample	Number of samples (n) tested	Number of samples (n) positive for					
		MDV	ALV	REV	MDV+ALV	ALV+REV	MDV+ALV+REV
Tissue / Visceral tumors	39	2	11	2	8	1	2
Feather follicle	8	4	-	-	-	-	-
Blood sample	4	-	2	-	-	1	-
Total	51	6	13	2	8	2	2

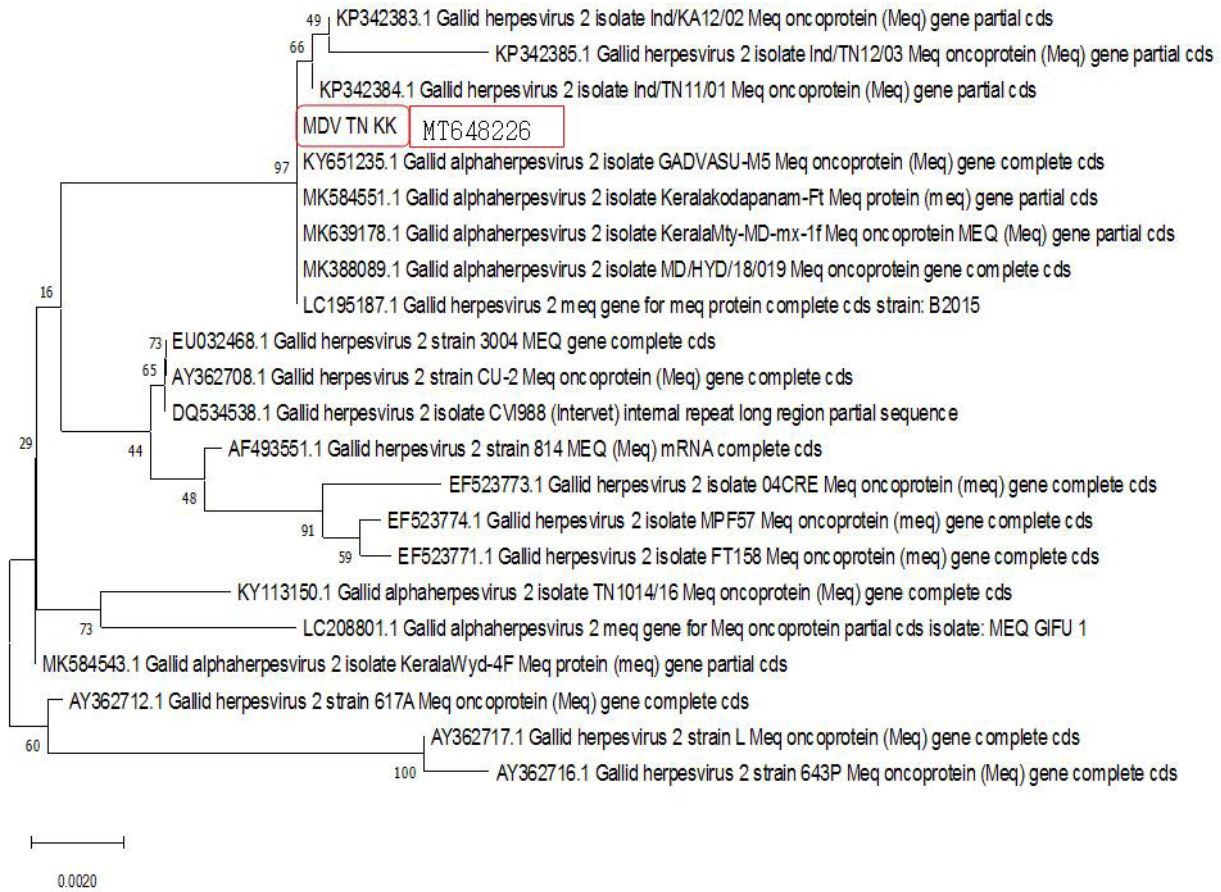


Fig. 3. Phylogenetic analysis based on partial *meq* gene sequence of MDV by neighbour joining method using MEGA version 11.0.10 (bootstrap 1000).

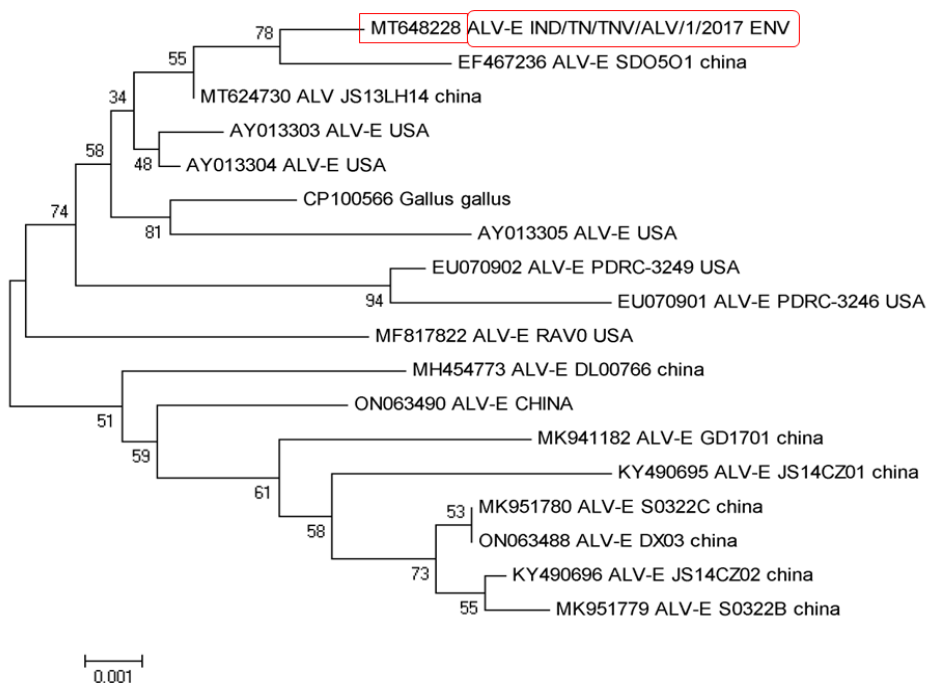


Fig. 4. Phylogenetic analysis of ALV sequence by neighbour joining method using MEGA version 11.0.10 (bootstrap 1000).

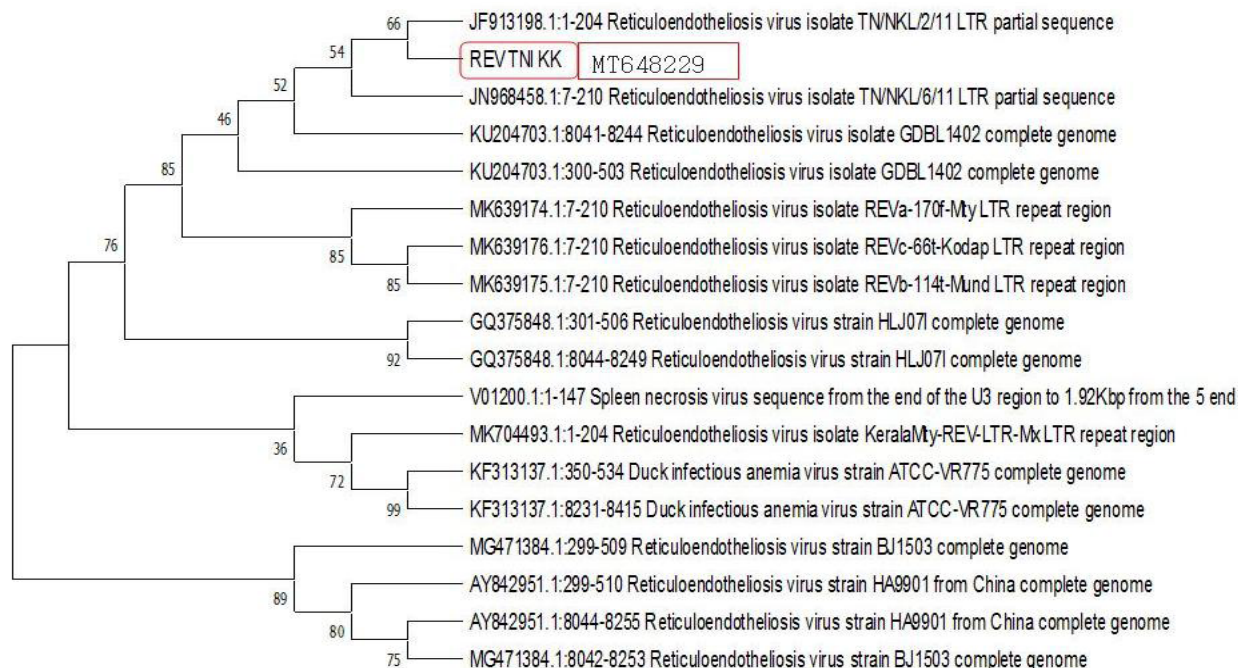


Fig. 5. Phylogenetic analysis of REV LTR sequence by neighbour joining method using MEGA version 11.0.10 (bootstrap 1000).

(GenBank accession number: MT648229) in the current study showed significant close relatedness of present isolate with the earlier known Tamil Nadu isolate with bootstrap value of 66% (Fig. 5). Though sporadic and subclinical, occasionally REV genomic sequences can be integrated into MDV genome in cases of mixed infections. This raises the possibility that pathogenicity of MDV could be altered or modified (Fadly and Venugopal 2008). Further, Sun *et al.* (2017) proved that experimental co-infection of MDV and REV potentially poses a serious threat to the affected chicken due to increased disease severity and reduced vaccinal protection against MD.

In conclusion, the molecular detection and differentiation of these multiple viral infections in native chicken by multiplex PCR approach will help in rapid differential diagnosis. Further, sequence analyses revealed that the MDV and REV strains detected from native chicken in the current study have the close proximity to the isolates circulating among the commercial chicken of Tamil Nadu. This knowledge on molecular characterisation of these circulating viruses will help in the control and eradication programmes as to will mitigate the economic losses in backyard poultry farming.

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