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Characterisation and phylogenetic analysis of repetitive DNA sequences in the *BamH1-H* region of Marek's disease virus from turkeys: Implications on viral pathogenicity and vaccine efficacy

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Abstract

Marek's disease virus (MDV) is a highly pathogenic alphaherpesvirus that causes paralysis and lymphoproliferative disease in birds, posing a significant threat to the poultry industry. Though initially described in chicken, the infection is now common in other species of poultry including turkeys and quails. This study investigated MDV infection in turkeys by screening 57 tissue samples. Out of these, 26 samples tested positive. The primary focus of the study was to characterise the 132 bp tandem repeats within the BamH1-H region of the MDV genome, the number of which has been previously correlated with the virulence of MDV. Each MDV-positive sample was found to contain two copies of the 132 bp repeats. Among the positive samples, two representative samples were sequenced and further analysis revealed high per cent similarity among each other. A phylogenetic analysis was conducted, providing insights into the evolutionary relationships of the field isolates. The findings of this study might contribute to a deeper understanding of MDV and in assisting future epidemiological studies and vaccine development strategies.

Keywords: *Marek's disease, 132* bp *repeats, molecular characterisation, phylogenetic analysis*

Marek's disease (MD) in poultry is an infection bearing great economic significance and is caused by Marek's disease virus (MDV), an oncogenic alphaherpesvirus (Churchill and Biggs, 1967). It was first identified in the early 20th century (Marek, 1907) and since then MD has evolved from a mild neuropathy to a devastating disease causing significant economic losses to the industry globally (Schat and Nair, 2013). The virus's ability to induce tumours and cause immunosuppression which can lead to increased mortality and decreased productivity in infected flocks are the main concerns faced by the poultry industry (Baigent and Davison, 2004).

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The MDV genome is a linear double-stranded DNA that features a unique long (U_L) region and a unique short (U_S) region, both of which are flanked by inverted repeat sequences internally (IR_L and IR_S) as well as at the terminals (TR_L and TR_S) (Fukuchi *et al*., 1984; Osterrieder and Vautherot, 2004).The molecular identification and confirmation of MDV are primarily achieved by targeting the *BamHI-H*-specific 132 bp repeats, which has been shown to be more effective compared to conventional virus isolation methods (Zhu *et al*., 1992; Sadeghi *et al*., 2006; Lebdah *et al*., 2017; Sun *et al*., 2017; Abd-Ellatieff *et al*., 2018). Maotani *et al*. (1986), Ross *et al*. (1993) and van Iddekinge *et al*. (1999) reported that the number of 132 bp repeats in the *BamH1-H* region of TR_S and IR_L might vary, and this variation could affect gene regulation.

However, the significance of this region in MDV pathogenesis was first recognised when it was observed that attenuation through cell culture passage led to an increase in the number of these 132 bp repeats (Silva and Witter, 1985). Research by Maotani *et al*. (1986) and Kanamori *et al*. (1986) also indicated that non-oncogenic MDV DNA has multiple copies of these tandem direct repeats. This finding is further supported by the presence of multiple copies of 132 bp repeats in apathogenic MDV 1 strain CVI 988 (Becker *et al*., 1993).

Zelnik (2003) and Silva and Gimeno (2007) reported that an increase in the number of 132-bp repeats is not the primary factor for the attenuation of the virus. On the other hand, Kalyani *et al*. (2011) demonstrated through their research that most of the outbreaks in vaccinated poultry flocks are due to MDV strains with a lower number of these repeats. This highlights the complex role of the 132 bp repeats in the MDV's genome. Thus, understanding these repeats might provide insights into the mechanisms of MDV evolution and this could pave the way to the development of more effective control strategies against this pathogen.

Vaccination has been the main strategy to control MD for several decades, significantly reducing the incidence of the disease. The protection provided by the current vaccines is often unsatisfactory because the ongoing viral evolution has led to increased virulence (Witter, 1997). Apart from that several other factors could also contribute significantly to the evolution of MDV virulence (age of the bird, immunosuppressive diseases). This calls for a more in-depth understanding of the genetic and molecular properties of MDV. Hence the present study was envisaged with the objective of detecting MDV infection in turkeys of Kerala as well as to characterise the virus's molecular features and evolutionary relationships.

Materials and methods

Sample collection:

Biomaterials were collected from suspected MD outbreaks at the District Turkey Farm in Kollam, Kerala and one sample from the University Poultry and Duck Farm (UPDF) in Mannuthy. A total of 57 tissue samples were gathered and stored at -20°C until DNA extraction for molecular diagnosis.

PCR Amplification and Sequence Analysis for MDV Detection and Characterisation:

Tissue samples were first homogenised under sterile conditions using mortar and pestle after which it was incubated along with proteinase k and a lysis buffer at 56°C overnight to facilitate complete lysis of the tissue homogenate.

The samples were processed for DNA extraction on the next day using Qiagen DNeasy blood and tissue kit, the extracted DNA was then stored in an elution buffer at -20°C until use.

A PCR protocol based on primers (Table 1) flanking the 132 bp repeats (Becker *et al*. 1992) located within the *BamH1-H* region of pathogenic MDV was standardised using a positive DNA template available at the Department of Veterinary Microbiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala. The direct primer is positioned 65 bp upstream of the 132 bp repeats, while the reverse primer is situated 105 bp downstream. In the case of a single 132 bp repeat, the anticipated amplified DNA band size is 302 bp, while for a double 132 bp repeat, it is 434 bp.

The PCR conditions included an initial denaturation at 94˚C for 1 min, followed by 31 cycles of denaturation at 94˚C for 1 min, annealing at 55˚C for 10 sec, elongation at 72˚C for 1 min and a final elongation at 72˚C for 10 min.

The PCR products were resolved using 1.5% (w/v) agarose gel (Sisco Research Laboratories Pvt. Ltd.) incorporating ethidium bromide at 0.5 µg/ml (Sambrook and Russell, 2001). The size of the DNA fragments were determined by comparing their migration distances with a 100 bp DNA ladder (Thermo Scientific Inc).

The representative amplicons were sequenced at Eurofins India Private Limited, Bangalore using Sanger's dideoxy nucleotide chain termination method. Both forward and reverse primers were used for bi-directional

Table 1. Primers for amplification of 132 bp repeats in the *BamH1-H* region

Sl. No.	Primer ID	Sequence (5'-3')	Amplicon size	Reference
1	*BamH1* (F)	TACTTCCTATATAGATTGAGACGT	434 bp	(Becker *et al*., 1992)
2	*BamH2* (R)	GAGATCCTTAAGGTTAATATA		

sequencing. The obtained sequence of each gene product was aligned and the NCBI BLAST tool was used to analyse the similarity of the products obtained with other published sequences available on the NCBI database.

***Phylogenetic analysis*:**

Reference sequences of the *BamH1-H* repeat regions from various MDV pathotypes were obtained from NCBI for analysis, with the details provided in Table 2. These sequences and those generated in the present study were aligned using CLUSTAL W. A phylogenetic tree was constructed using MEGA 11 with the Neighbour-Joining method, employing 1,000 bootstrap replicates based on the maximum composite likelihood approach. Positions with gaps and missing data were omitted from the analysis to improve accuracy.

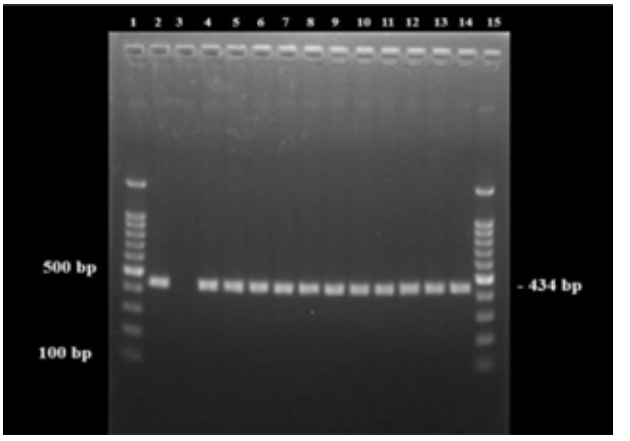

Fig. 4. Agarose gel electrophoresis pattern of MDV 132 bp repeats specific PCR Products (~ 434 bp in case of double 132 bp repeats)

Lane 1 & 15 – 100 bp ladder Lane 2 – Positive control
Lane 3 – Negative control Lane 4 to 14 - Samples

Results and discussion

Out of the 57 tissue samples, 26 tested positive for MDV by PCR. A 434 bp amplicon was generated by all positive samples (Fig. 1), suggesting the presence of a double 132 bp repeat. The BLASTn search of the sequenced product from representative samples confirmed their identity and the obtained sequences are deposited in the NCBI database with accession numbers PQ227826 and PQ227827.

These findings align with those observed by Kalyani *et al*. (2011), who characterised the 132 bp repeats in MDV and highlighted their potential as molecular markers for distinguishing between pathogenic and non-pathogenic strains. They arrived at the conclusion that more pathogenic strain have lesser copies of 132 bp repeats. This inference is further supported by earlier studies, including those by Maotani *et al*. (1986) and Kanamori *et al*. (1986), who demonstrated the presence of multiple copies of 132 bp repeats in non-oncogenic strains. Similar findings were reported by Becker *et al*. (1993) during their analysis of the repeat regions in the MDV serotype 1 vaccine strain CVI988. These studies collectively emphasise the critical role of these repeats in the characterisation of MDV.

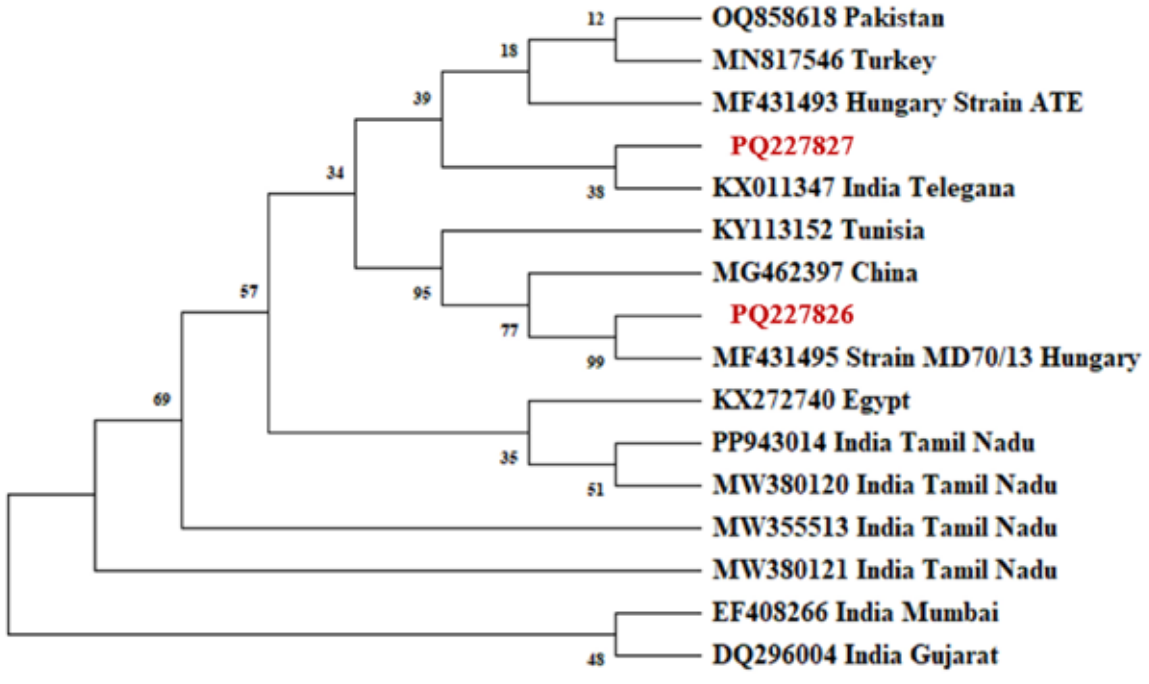

Fig. 2. Phylogenetic tree depicting the evolutionary relationship of field isolates with reference sequences obtained from the NCBI database

Having identified the presence of a 434 bp amplicon suggestive of double 132 bp repeats in the *BamH1-H* region of the MDV isolate, the study further sought to understand the genetic relationship of this isolate with other known MDV strains. A phylogenetic tree was constructed (Fig. 2) based on the nucleotide sequences of the *BamH1-H* region.

The phylogenetic tree constructed from the *BamH1-H* region sequences of MDV isolates and reference sequences from GenBank revealed significant insights into the genetic relationships of the isolates under study. Notably, the two isolates from the study, LMDT8 and LMDT24, were positioned within distinct clades, reflecting their genetic divergence and possibly different evolutionary origins.

The phylogenetic analysis of the isolates LMDT8 and LMDT24 reveals distinct evolutionary pathways across diverse geographical regions. Isolate LMDT8 clusters with strains from Hungary, Turkey, and Pakistan, suggesting moderate genetic similarity and potentially shared evolutionary origins. In contrast, LMDT24 demonstrates a strong genetic relationship with isolates from Hungary and Egypt, supported by high bootstrap values, indicating a likely common ancestry or recent genetic exchange.

The separation of LMDT8 and LMDT24 into different clusters indicates their distinct evolutionary paths even though they originated from the same study. The observed diversity in the phylogenetic tree reflects a high level of genetic variability in MDV strains worldwide. Although several studies in India have characterised MDV strains from chickens, there is a notable gap in research focusing on MDV infections in turkeys. Given that MDV evolve under different selection pressures, the findings of this study will enhance the current understanding of the MDV genome affecting the turkey flocks. Identifying any genetic variations or unique molecular markers associated with MDV in turkeys will be critical for refining diagnostic tools, improving control strategies, and guiding vaccine development tailored for these avian species.

Conclusion

In conclusion, the genetic diversity identified in this study, particularly in the *BamH1-H* region, highlights the need for continuous monitoring of MDV strains. Vaccination strategies may need modification to ensure effective management of Marek's disease in poultry. As MDV continues to evolve, it is critical to ensure that vaccination programs are adaptable and capable of addressing the challenges posed by emerging variants.

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Conflict of Interest:

The authors declare that they have no conflict of interest.

References

Abd-Ellatieff, H. A., Abou Rawash, A. A., Ellakany, H. F., Goda, W. M., Suzuki, T. and Yanai, T. 2018. Molecular characterisation and phylogenetic analysis of a virulent Marek's disease virus field strain in broiler chicken in Japan. *Avian Pathol*. **47**: 47-57.

Baigent, S.J. and Davison, T.F. 2004. Marek's disease virus: biology and life cycle. In: Davison, F. and Nair, V. (ed.), *Marek's Disease, an Evolving Problem*. Elsevier Academic Press, Oxford, pp. 62-77.

Becker, Y., Asher, Y., Tabor, E., Davidson, I., Malkinson, M. and Weisman, Y. 1992. Polymerase chain reaction for differentiation between pathogenic and non-pathogenic serotype 1 Mareks disease viruses (MDV) and vaccine viruses of MDV-serotype 2 and 3. *J. Virol. Methods.* **40**:307-322.

Becker, Y., Tabor, E., Asher, Y., Davidson, I., Malkinson, M. and Witter, R. L. 1993. PCR detection of amplified 132 bp repeats in Marek's disease virus type 1 (MDV-1) DNA can serve as an indicator for critical genomic rearrangement leading to the attenuation of virus virulence. *Virus Genes*. **7**: 277-287.

Churchill, A.E. and Biggs, P.M. 1967. Agent of Marek's disease in tissue culture. *Nature.* **32**: 528-530.

Fukuchi, K., Sudo, M., Lee, Y. S., Tanaka, A. and Nonoyama, M. 1984. Structure of Marek's disease virus DNA: detailed restriction enzyme map. *J. Virol*. **51**: 102-109.

Kalyani, I. H., Joshi, C. G., Jhala, M. K., Bhanderi, B. B. and Purohit, J. H. 2011. Characterization of 132 bp repeats BamH1-H region in pathogenic Marek's disease virus of poultry in Gujarat, India, using PCR and sequencing. *Indian J. Virol.* **22**: 72-75.

Kanamori, A., Nakajima, K., Ikuta, K., Ueda, S., Kato, S. and Hirai, K. 1986. Copy number of tandem direct repeats within the inverted repeats of Marek's disease virus DNA. *Biken J.* **29**: 83-89.

Lebdah, M. A., Nassif, S. A., Shahein, A. M. and El-Basrey, Y. F. 2017. Isolation and identification of very virulent strains of Marek's disease virus from MDV-vaccinated flocks in egypt. *Zag. Vet. J.* **45**: 197-205.

Maotani, K., Kanamori, I. K., Ueda, S., Kato, S., and Hirai, K. 1986. Amplification of a tandem direct repeat

within inverted repeats of Marek's disease virus DNA during serial in vitro passage. *J. Virol*. **58**:657-660.

Marek, J. 1907. Multiple Nervenentzündung (Polyneuritis) bei Hühnern. *Dtsch*. *Tierärztl*. *Wschr*. **32**: 417-421.

Osterrieder, N. and Vautherot, J.F. 2004. The genome content of Marek's disease-like viruses. *Curr. Top. Microbiol.* 17-31.

Ross, N., Binns, M. M., Sanderson, M. and Schat, K. A. 1993. Alterations in DNA sequence and RNA transcription of the BamHI-H fragment accompany attenuation of oncogenic Marek's disease herpesvirus. *Virus Genes.* **7**:33-51.

Sadeghi, M. R., Ghorashi, S. A., Kargar, M. R., Morshedi, D., Salehi, T. R. and Ghaemmaghami, S. S. 2006. Polymerase chain reaction for the detection and differentiation of Marek's disease virus strains MDV-1 and HVT. *Iran J. Vet. Res*. **7**: 17-21.

Sambrook, J. and Russell, D. W. 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Schat, K. A., and Nair, V. 2013. Neoplastic diseases: Marek's disease. In: Swayne, D. E., Glisson, J. R., McDougald, L. R., Nolan, L. K., Suarez, D. L. and Nair, V. L. (Ed.) *Diseases of Poultry*. (13th Ed.). Wyley-Blackwell, Hoboken, New Jersey, pp. 515-552.

Silva, R.F. and Gimeno, I. 2007. Oncogenic Marek's disease viruses lacking the 132 base pair repeats can still be attenuated by serial in vitro cell culture passages. *Virus Genes*. **34**: 87-90.

Silva, R. E, and Witter, R. L. 1985. Genomic expansion of Marek's disease virus DNA is associated with serial in vitro passage. *J. Virol***. 54**: 690-696.

Sun, G. R., Zhang, Y. P., Lv, H. C., Zhou, L. Y., Cui, H. Y., Gao, Y. L., Qi, X. L., Wang, Y. Q., Li, K., Gao, L. and Pan, Q. 2017. A Chinese variant Marek's disease virus strain with divergence between virulence and vaccine resistance. *Viruses*. **9**: 71.

van Iddekinge, B. H., Stenzler, L., Schat, K. A., Boerrigter, H. and Koch, G. 1999. Genome analysis of Marek's disease virus strain CVI-988: effect of cell culture passage on the inverted repeat regions. *Avian Dis*. **43**: 182-188.

Witter, R. L. 1997. Increased virulence of Marek´s disease virus field isolates. *Avian Dis*. **41**: 149-163.

Zelník, V. 2003. Marek's disease virus research in the post-sequencing era: new tools for the study of gene functions and virus–host interactions. *Avian Pathol*. **32**: 323-333.

Zhu, G. S., Ojima, T., Hironaka, T., Ihara, T., Mizukoshi, N., Kato, A., Ueda, S. and Hirai, K. 1992. Differentiation of oncogenic and nononcogenic strains of Marek's disease virus type 1 by using polymerase chain reaction DNA amplification. *Avian Dis*. **36**: 637-645.

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