Characterization of Marek's disease virus and phylogenetic analyses of *meq* gene from an outbreak in poultry in Meghalaya of Northeast India

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Abstract The aim of the present study was to characterize the virus from the lesions and histopathology of organs associated with mortality in Kuroiler (dual purpose variety of poultry developed and marketed by Keggfarms Pvt. Ltd, India) birds suspected of Marek's disease. Among 1047 birds from two farms of different location with 5.5 and 34% mortality, two types of lesion were observed in post mortem examination; tumors in vital organs—liver, spleen, kidney, lung and ovaries and generalized small nodular tumour in the abdominal cavity. Molecular characterization based on detection of *ICP4* gene showed the presence of Marek's disease virus (MDV) from tissues and cell culture adapted isolates in Madin Darby Canine Kidney cell lines. Histopathological examination revealed multinucleated immature lymphoid cells infiltration in the organs. Phylogenetic analysis of the isolates based on *meq* gene showed the isolates belongs to cluster I genotype of MDV. This is for the first time the MDV virus is characterized from an outbreak in the poultry flock in farmer's field affecting production in Meghalaya state of North east India.

Keywords Marek's disease · Kuroiler · Oncogenic virus · Serotype · Genotype

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Introduction

The oncogenic viral diseases of poultry—Marek's disease virus (MDV), Avian Leukosis virus (ALV) and Reticuloendotheliosis virus (REV) are responsible for economic loss due to both mortality and depressed performance [15]. MDV continues to pose severe threats to the poultry industry around the world with increasing reports of vaccination breaks and emergence of more virulent pathotypes [21]. Evolution of virus does occur at alarming rates in the poultry houses regardless of the vaccination status [14]. The gross lesions caused by avian oncogenic virus infections often overlap, and diagnosis based on gross lesions is difficult, necessitating specific laboratory diagnosis [5]. Histopathological examination as a confirmatory diagnostic method in tumor induced by viruses in chicks has been considered one of the main procedures for differentiation of the causative agents for tumors in chickens [24]. Molecular techniques such as PCR is use for the diagnosis of avian oncogenic viruses because it enables the detection of multiple viral infections [3, 20] but virus isolation is considered the 'gold standard' for diagnosis and is usually the starting point for further detailed studies [14]. Though the MD vaccines are capable of preventing lymphoma formation and clinical disease, do not prevent superinfection by pathogenic MDV strains [15]. Therefore, detection and elimination of infected birds form the basis for diagnosis and eradication programmes for oncogenic viruses [16]. In the present study, we investigated the samples from an outbreak, the lesions suspected of oncogenic viruses in poultry by histopathology of organs involved and molecular characterization of virus to understand the strain of virus present in the poultry flock in the region.

Materials and methods

Case history

Cases of poultry mortality from two locations in Ri Bhoi district of Meghalaya occurs in the month of January and March 2015, respectively. In the first case, 55 numbers of 8 months old dual purpose Kuroiler died out of 1000 numbers (5.5% mortality) within 3 days. The dead birds (5 nos) were brought for examination and seek advice for treatment of the flock. In second case, 16 birds of 11 months old Kuroiler birds died out of 47 numbers (34% mortality) and 4 numbers of carcasses were brought for diagnosis with the farmers reporting of an adult laying birds died without apparent sicknesses. The chicks were brought from reputed commercial firms with vaccination done against MDV on day old chicks.

Study location

The investigation was done at Animal Health Division, Indian Council of Agriculture Research-Research Complex for North Eastern Hill Region, Umiam, Meghalaya. On opening the carcasses were found to have developed tumours in liver, lungs, kidney, spleen, ovaries and some with generalized small nodules scattered in the abdominal cavity and enlarged sciatic nerves (Fig. 1).

Tissue samples

The tissues of kidney, liver, ovary, spleen and lungs having lesions were collected during post mortem examination and divided into two parts. One part is kept at 10% formalin for histopathology and another part was triturated and 10% suspension was made with 1X PBS (pH 7.2–7.4) for virus isolation and molecular detection and characterization.

Molecular detection of oncogenic viruses

Molecular detection of MDV was done by detection of *ICP4* gene with the primers sequences-forward 5'-GGAT CGCCCACCACGATTACTACC-3' and reverse 5'-ACTG CCTCACACAACCTCATCTCC-3' [10] and *Meq* gene primer, forward—5'-ATGTCTCAGGAGCCAGAGCCGG CGCT-3' and reverse—5'-GGGGCATAGACGATGTGCT GCTGAG-3' [11]. The DNA from the tissue was extracted using QIAamp viral DNA kit (Qiagen, GmbH, Hilden, Germany) and used for detection of MDV. Amplification was performed by initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 35 s for *ICP4* gene and 58 °C for 45 s for *meq* gene and extension at 72 °C for 30 s with a

final extension at 72 °C for 7 min. The molecular detection of other oncogenic viruses—REV and ALV by RT-PCR was done by detection of REV 5'*LTR* with the primer P5 (5'-ACC TAT GCC TCT TAT TCC AC-3') and P6 (5'-CTG ATG CTT GCC TTC AAC-3') and *env* genes with primer P3 (5'-TGA CCA GGC GGC CAA AAC C-3') and P4 (5'-CGA AAG GGA GGC TAA GAC T-3') as per [22, 23] respectively. While ALV subtype group (A–J) detection was done as per [12]. The total RNA was extracted from tissue samples by using a QIAamp viral RNA minikit (Qiagen, GmbH, Hilden, Germany). Extracted RNA was reverse transcribed using a RevertAid first-strand cDNA synthesis kit (Thermo Scientific, USA) employing a random hexamer primer following manufacturers' protocol. Amplification was performed by initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 35 s for ALV genes and 52 °C for 30 s for 5'*LTR* and *env* gene and extension at 72 °C for 30 s with a final extension at 72 °C for 7 min.

Visualization of amplified product

Amplicons were analyzed by electrophoresis through 1.5% agarose-TAE (40 mM Tris-acetate, pH 8.0, 1 mM EDTA) gel, stained with ethidium bromide and visualized in gel documentation system (DNR MiniLumi, Israel).

Sequence analysis

The amplified PCR products were purified by PCR purification kit (Qiagen, GmbH, Hilden, Germany) according to manufacturer's instruction. Purified PCR products ligated into pMD20 cloning vector (Takara, Japan) and transform into *E. coli* DH5 α competent cells (Invitrogen, Germany). Purified plasmid from clones were subjected to sequencing from both ends using universal primer M13 forward and T-7 promoter on 24 capillary ABI 3500xL Genetic Analyzer automatic sequencer (Applied Biosystems). Nucleotide sequences were assembled by SeqMan analysis software (DNASTAR, Inc., Madison, WI, USA) and aligned in Clustal omega (<http://www.clustal.org/>) software. Similarities in nucleotide sequences were evaluated using the Basic Local Alignment Search Tool (BLAST).

Phylogenetic analysis

Nucleotide sequences of the fragments were aligned using the ClustalW program (www.clustal.org/). Further phylogenetic analysis was performed with the neighbor-joining method using the MEGA5 program (www.megasoftware.net), and evolutionary distances were calculated using the

Fig. 1 Representative post mortem lesions. 1 generalized nodular lesion, 2 sciatic nerve enlargements, 3 kidney tumor, 4 lung tumor

Tajima Nei method. The robustness of the phylogenetic analysis and the significance of branch order were determined using the bootstrapping method based on 1000 replicates.

Histopathology of tissue/organs

The tissues in 10% buffered formalin were processed for histopathological tissue section and preparation of slides as per the standard protocol. The tissue section were stained by H&E stain and visualized under light microscope fitted with imaging software.

Virus adaptation into cell culture

The representative samples from both the places were taken and made to 10% suspension of tissue triturate was filtered through 0.45 μ m syringe filter and added to MDCK cell culture by co-cultivation method with initial 5 blind passages. Subsequent passages were checked for the presence of changes in the cell as well as PCR detection of specific genes for the confirmation of virus in each passage.

Results and discussion

PCR detection of oncogenic viruses

The PCR amplification of 318 bp product from all the tissue samples and subsequent cell culture adapted samples indicated the presence of *ICP4* gene of MDV in the samples. The amplification of 1062 bp product for *Meq* gene was also positive from the samples indicated the virus is of virulent serotype 1. The PCR amplification for *5'LTR* and *env* genes of REV was negative from all the samples while amplification of 265 bp product indicated the detection of subgroup E of ALV. The presence of MDV has been reported from various states of India but limited information on its characterization. Sporadic outbreaks of MD from vaccinated flocks [1, 8] and molecular methods for differential detection of avian oncogenic viruses—ALC or REV in poultry and turkeys has been reported [8]. Our result showed the presence of MDV-1 in Meghalaya. Amplification of 318 bp *ICP4* gene was in agreement with [10] who reported similar finding from field samples indicating the MDV-1. The presence of REV in the samples was also investigated but could not amplify any target

genes. However, the amplification of 265 bp product indicated the detection of subgroup E of ALV. Subgroup E is an endogenous leukosis virus, which has low or no pathogenicity to chickens directly [18] but studies have shown that chickens infected with ALV-E remained viremic with exogenous virus longer and developed neoplasm at a much higher frequency than did the control chickens not infected with ALV-E. So all the common ALV subgroups needed to be eradicated from the chicken breeder flocks [16].

Phylogenetic analysis and determination for virulence

The sequences of *Meq* gene were analysed and submitted to DNA Data Bank of Japan (DDBJ) and accession number LC195187 and LC195188 were assigned for the isolates. *Meq* gene based phylogenetic analysis of MDV isolates (Fig. 2) were performed to investigate genetic relationship of the isolates in our study with 30 reference *meq* gene sequences from gene bank from different geographical locations (Supplementary material: Table 1) which revealed three major clusters. Both isolates were found to belong to cluster I with other nine isolates from North India and one isolate from Hungary and amino acid sequence analysis revealed both the isolates possess 339 amino acids and 100% matching with AY571784—ATE strain from Hungary (Fig. 3). Both the isolates have 5 repeat sequence of 4 prolines (PPPP) at 152–155, 175–178, 191–194, 216–219 and 232–235 positions (Supplementary material: Table 2). The phylogenetic analysis of *meq* gene of the two isolates along with 30 reference strain from various geographical areas revealed three major clusters with the two isolates in study clustered with the north India isolates and one isolate from Hungary into cluster I while the south Indian isolates were in cluster III along with one isolate each from China, Russia, USA and commercial vaccine strain. Most of the strains from China and USA used in the study were in cluster II. While *meq* gene detection indicates the pathogenicity of the virus, the number of repeat sequences of four prolines (PPPP repeats) in the *meq* gene (overall range 2–8) have been reported to correlate with MDV virulence and oncogenicity [19] with the most pathogenic isolates having the fewest number of repeats [17]. Compared with 30 reference strains, both the isolates have 5 PPPP repeats similar to the six isolates from north India [9] and one each from Hungary and South India indicating the virulence potential. The isolates in cluster II have 2–4 PPPP repeats while the isolates in cluster III have PPPP repeats ranges from 4 to 7 probably indicating the level of pathogenic potential of the isolates in each clusters. The increased virulence of MDV strains depend on the interruption of the repeats of prolines at the second

positions with the most virulent MDV having the greatest number of interruptions at position 153, 176 and 276 [19].

Histopathological findings

Many multinucleate cells with immature lymphoid cells infiltration present in the compartment of the organs (liver, spleen, Kidney and ovaries) were found in the histopathological diagnosis, ultimately destroying the tissue structure correspond to the classical lesions of tumors found in Marek's disease (Fig. 4). Based on histopathology, the presence of immature lymphoid cells infiltration into the organs corresponds to the classical lesions of tumors found in Marek's disease. The cytological picture of MDV could be typical, while histological differentiation between ALV and REV may not be possible [4]. However, histopathology examination as a confirmatory diagnostic method in tumor induced by viruses in chicks has been considered one of the main procedures for differentiation of the causative agents for tumors in chickens [25].

Virus adaptation

The virus adaptation was observed with the changes in the cells start appearing after five blind passages. The cytopathic changes observed includes rounding and sloughing of cells, formation of plaque which starts 72 h post infection (PI) to sloughing of cells by 120 h. The adaptation of virus into the MDCK cells was confirmed by cytopathic changes like plaque formation and confirmation by PCR amplification of target genes.

Conclusion

In the present study, to demonstrate the genomic features of MDV circulating in Meghalaya, *meq* gene was used to analyze the virulence from two isolates representative of two positive flocks and compare with the published sequences from the gene bank. It was found that both the isolate were clustered in the same group with the north Indian isolates with similar number of PPPP repeats perhaps indicate the presence of similar virulent strains circulating in the region. The vaccines are frequently used in broiler and layers chickens at 1-day old but the pathotypes referred to as very virulent (vvMDV) or very virulent plus (vv+MDV) were isolated from flocks vaccinated with different vaccination regimes and vaccination does not prevent superinfection, replication and shedding of virulent challenge viruses [6, 7], so chickens can potentially be infected simultaneously with both vaccine and virulent MDV strains [2] and the role of vaccines themselves in assisting the drive towards increasing virulence has not

Fig. 2 Phylogenetic tree of MDV based on Meq gene

Fig. 3 Amino acid alignment of MDV Meq gene sequences

Fig. 4 H&E stain showing infiltration of lymphomas into the **a** glomeruli and tubules of kidney, **b** liver and **c** alveoli of lung tissue. (10X)

been ruled out [13]. The inability of the vaccine to protect chickens against overt clinical signs following field infection has also been ascribed to early exposure [26]. Therefore MD could again emerge as a major economic problem for the poultry industry. Future research aim at ways to improve vaccine induced protection or inducing sterile immunity to stop the evolution of the virus if possible could be a solution to MDV infection affecting poultry.

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