Characterization of 132 bp Repeats *Bam*H1-H Region in Pathogenic Marek's Disease Virus of Poultry in Gujarat, India, Using PCR and Sequencing

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Abstract A total of 34 clinical samples and four Marek's disease virus (MDV) vaccines were tested using primer *Bam*H1/*Bam*H2 in layer birds of poultry. Out of 34 samples tested for detection of MDV, 32 samples produced approximately 434 bp product. All the three HVT vaccines as well as SB-1 (MDV-2) vaccine failed to produce the expected amplicons, thereby proving negative for the targeted 132 bp repeats of MDV genome by the primers *Bam*H1/*Bam*H2. Resultant PCR products of the field samples were purified and sequenced and resulted in 378 bp long sequences. PCR was found very satisfactory in detecting the presence of MDV either in feather follicle or in tissue samples. Sequencing study has proved beyond doubt that the two representative samples contained two 132 bp repeats indicating the virulent nature of the field virus.

Keywords Marek's disease virus · PCR · Sequencing

One of the most economically devastating infectious diseases of poultry is Marek's disease (MD), caused by Marek's disease virus (MDV), an oncogenic avian herpesvirus. MD in chickens is commonly characterized by a generalized lymphomatosis involving lymphocytic

infiltration of nerves and other organs. Clinical manifestations can be numerous and varied, including paralysis, skin lesions, atrophy of the thymus and bursa of fabricius, immuno-suppression, and high mortality. MD commonly appears in 3–4 week old chickens and gradually builds to a peak between 12 and 30 weeks of age. Polymerase chain reaction (PCR) has emerged in recent years as an additional diagnostic tool offering the advantages of serotype specificity and the ability to differentiate between vaccinal and field strains of MDV serotype-1 [4, 8, 12]. The importance of *Bam*HI-H region in the pathogenesis of MDV was initially pointed out; when it was shown that there was an increase in the number of copies of 132 bp repeats within the *Bam*HI-H region of inverted repeats (IR) as a result of attenuation by cell culture passage [9]. A PCR based on primer flanking 132 bp repeat sequence located within the *Bam*HI-H fragment in pathogenic MDV-1 DNA was developed [1]. The primers were selected from the nucleotide sequence flanking 132 bp repeat sequence located within the *Bam*HI-H fragment published [3]. The direct primer is located 65 bp 5' to the tandem 132 bp repeats; the reverse primer is 105 bp downstream of 132 bp repeats. Altogether, the expected amplified DNA band size in case of double 132 bp repeat is 434 bp. PCR assays were also used to amplify a region of the ICP4 gene in layer birds of poultry by primer set M1.1/M1.8 indicating that the MDV is prevalent in Gujarat [5]. Presence of virulent/very virulent MDV serotype 1 strains in the infected liver tissues were also reported in the Indian poultry flocks [7].

The technology of nucleic acid amplification is now widely applied in the diagnosis of poultry diseases, MDV infection has been known to occur in the state and country for past 35 years or more, work involving detection of MDV by PCR and other aspects like genetic characterization by DNA sequencing has not apparently been carried out. Keeping

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above facts in view, present study was under taken for the detection of target nucleotide sequences flanking the 132 bp repeats in *Bam*H1-H and *Bam*H1-D fragments of the MDV by PCR and sequencing of PCR product from clinical samples of chicken in layer birds from the selected areas of Gujarat state, India.

A total of 34 clinical samples for MDV detection by PCR were collected from 27 layer birds. Minimum of five feathers were collected from an individual bird. Samples were collected from different commercial poultry farms (16 samples from 12 birds, comprising 8 feather samples and 8 spleen samples) located in Anand district as well as from CPRS, A.A.U., Anand (18 samples from 15 birds, comprising of 9 feather samples and 9 spleen samples), with a history of mortality and postmortem lesions suggestive of MD. One Lyophilized freeze-dried live HVT vaccine (HVT-126) obtained from the Division of Standardization, IVRI, Izatnagar, U.P. and three commercially available vaccine strains namely Cell free HVT vaccine, Cell associated HVT vaccine and Cell associated MDV-2 (serotype-2 MDV) vaccine obtained from Ventri Biologicals Ltd., Pune, India, were used as reference strains. Extraction of DNA from feather tips was carried out as per the procedure described by Handberg et al. [4]. DNeasy Tissue Kit (Catalog no. 69504, QIAGEN Pvt. Ltd) was used for extraction of DNA from tissue samples and vaccines. A PCR based on primer flanking 132 bp repeat sequence located within the *Bam*H1-H fragment in pathogenic MDV-1 DNA was developed [1]. The primers *Bam*H1 5' TAC TTC CTA TAT AGA TTG AGA CGT 3' and *Bam*H2 5' GAG ATC CTC GTA AGG TGT AAT ATA 3' were selected from the nucleotide sequence flanking 132 bp repeat sequence located within the *Bam*H1-H fragment published [3]. PCR was carried out in a final reaction volume of 25 µl using 200 µl capacity thin wall PCR tube containing 3 µl template DNA, 10 × PCR buffer (Sigma-Aldrich, USA), 25 mM MgCl₂, 200 µM of the four dNTPs, 10 pmol of each primer (Bangalore Genei, India), and 1U *Taq* DNA polymerase (Sigma-Aldrich, USA). The PCR tubes with all the components were transferred to thermal cycler (Eppendorf Master Cycler, Germany). The PCR was carried out with initial denaturation at 94°C for 1 min, followed by 31 cycles of denaturation at 94°C for 1 min, annealing 55°C for 10 s and extension 72°C for 1 min, and final extension at 72°C for 10 min. After amplification, 5 µl of the reaction mixture was electrophoresed in a 2% agarose gel, stained with ethidium bromide, and visualized by a Gel documentation system (SynGene, Gene Genius Bio Imaging System, UK).

Sequencing of *Bam*H1/*Bam*H2 primer amplified PCR products were carried out. (a) Two samples were selected from PCR product amplified by primer pair *Bam*H1/*Bam*H2 (b) BigDye® Terminatorv1.1Cycle Sequencing Kit

with following components. (i) Ready reaction premix (ii) Big dye sequencing buffer (5×) (iii) pGEM®-3Zf(+) double stranded control DNA template (c) Performance optimized polymer (POP-6™) (Part No. 402837 Applied Biosystems, USA) (d) Sequencing primer *Bam*H1 forward (Synthesized by Sigma-Aldrich, USA) (e) Ethanol (f) EDTA 125 mM (g) Sodium acetate (3 M) (h) 70% Ethanol (i) Formamide (Sigma-Aldrich, USA, Catalog No. F-9037). Cycle sequencing was performed following the instructions supplied along with BigDye® Terminatorv1.1 Cycle Sequencing Kit. The reaction was carried out in a final reaction volume of 20 µl using 200 µl capacity thin wall PCR tube. Composition of reaction mixture for cycle sequencing contains 4 µl of 2.5× ready reaction premix (final concentration 0.5×), 2 µl of 5× big dye sequencing buffer (final concentration 1×), 1 µl forward primer *Bam*H1 (3.2 pmol/µl), 5 µl PCR product (30 ng/µl), 8 µl deionized water with 20 µl final reaction volume. The tubes containing the mixture were tapped gently, spun briefly and then the tube with all the components was transferred to thermal cycler. The Cycling protocol for sequencing reaction was designed for 25 cycles with initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing 55°C for 10 s, extension 72°C for 4 min and the thermal ramp rate of 1°C per second. Before subjecting the extension products for electrophoresis through the POP-6™ polymer filled capillary of automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer, Applied Biosystems, USA), using BigDye® Terminator v3.1 Cycle sequencing kit (Applied Biosystems, USA). The unincorporated dye terminators were removed complete by ethanol precipitation in presence of EDTA and sodium acetate as follows. The 20 µl extension product was mixed thoroughly with 2 µl of 125 mM EDTA, 2 µl of 3 M sodium acetate and 50 µl of 100% ethanol. DNA was allowed to precipitate by incubating the mixture for 15 min at room temperature, the precipitated DNA was pelleted by centrifuging at 11,000 rpm for 45 min at 4°C, the pellet was washed with 70 µl of 70% ethanol by centrifuging at 11,000 rpm for 15 min at 4°C. Finally the pellet was dried and resuspended in 20 µl formamide. After then electrophoresis and data analysis were carried out on the ABI PRISM® 310 Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files. The nucleic acid sequence obtained from the 132 bp repeats PCR products were aligned with known sequences of 132 bp repeats of MDV genome available in Genbank®. The nucleotide sequences (excluding primer sequences) of both m3 and m4 samples 132 bp repeats located within *Bam*H1-H and *Bam*H1-D segment of viral genome amplified by PCR were aligned using the Clustal W programme [10].

Out of 34 samples (17 feather follicles and 17 spleen tissues) tested for detection of MDV, 32 samples (15

Fig. 1 Agarose gel electrophoresis pattern of MDV 132 bp repeats specific PCR Products (approximately 434 in case of double 132 bp repeats) amplified with primer pairs *BamH1/BamH1*. M: DNA molecular weight Markers, N: negative control, 1–3: spleen samples, H: HVT vaccine, 4–6: feather samples

samples were from commercial poultry farms and 17 samples were from the organized, CPRS farm) produced approximately 434 bp (double 132 bp repeats) amplicon (Fig. 1). Of the 17 feather follicle samples, 15 produced the targeted amplicons, whereas, all the 17 spleen samples produced the amplicons of about 434 bp. All the three HVT vaccines as well as SB-1 (MDV-2) vaccine failed to produce the expected amplicons, thereby proving negative for the targeted 132 bp repeats of MDV genome by the primers *BamH1/BamH2*. All the birds, which were showing the MD symptoms, were vaccinated on day one by HVT (MDV-3) vaccine, from different commercial vaccine producing companies. Moreover, the birds from commercial poultry farms were vaccinated with SB-1 (MDV-2) vaccine also. However, the birds of CPRS were vaccinated only with HVT vaccine. But the PCR amplified product from all the positive cases did not differ in the band size i.e. 434 bp irrespective of single or double vaccination status. Same way, PCR product either from feather follicle sample or spleen sample also did not differ in band size i.e. 434 bp. Hence it is logical to believe that all the positive samples by this primer pair had double 132 bp repeats. Differentiation of pathogenic and non-pathogenic serotype-1 MDV by amplification of the 132 bp tandem repeats within the MDV genome was carried out by Silva, [8]. Further similar observation was made, when DNA extracted from spleen and liver of MDV infected birds revealed dimmer tandem 132 bp repeats [1]. In five of the MDV-1 isolates, one DNA fragment was amplified. This fragment corresponded to an amplification of two tandem repeats of 132 bp yielding a DNA band of 453 bp (264 bp plus 189 flanking tandem repeats). These five isolates with two 132 bp repeats were from commercial flocks affected with number of lymphomas in the internal organs or from experimentally infected SPF chicken. To substantiate the

finding, Becker et al. [2] used the same PCR (i.e. amplification of 132 bp repeats) in MDV type-1 as an indicator for critical genomic rearrangement leading to the attenuation of virus virulence. In a similar study, Zhu et al. [12] differentiated the oncogenic and non-oncogenic strain of MDV-1 by primer chosen from the sequence within the long inverted repeats of MDV-1 DNA.

Considering the above facts, in present study PCR product amplified by a primer pair *BamH1/BamH2* was selected for sequencing analyses to know exactly whether the amplified fragment contained the 132 bp repeats and if so how many. So in present study both the samples resulted in 378 bp long sequences, the remaining length of bps were not showing high confidence level and hence were not used for further analysis study. In both the samples, two 132 bp repeats were found. Both sequences were submitted to Gene Bank, National Centre for Biotechnology Information, USA, with the accession no DQ296003 and DQ296004. The alignment of 378 bp sequence with previously published sequences of MDV isolates Md5, Md11, HPRS-16 and CDs of tumorigenicity associated m-RNA and CDs published from *BamH* gene family revealed that the two representative field isolates of present study shared maximum homology (100%) as expected, due to highly conserved region. The alignment of 378 bp sequence with Md5, Md11 and HPRS16 indicated that both the samples contained a genome (fragment) of MDV-1 only. The PCR product of *BamH1/BamH2* was specifically selected for sequencing study, because the part of MDV genome which is amplified with this set of primer exists only in MDV-1 (i.e. 132 bp repeats) and not in MDV-2 or MDV-3 and the results were as per the expectation and in accordance with the other published reports concerning the same (132 bp repeats) segment of MDV genome [3, 11]. The double 132 bp repeats as observed in the present study has also been reported in a genome of vvMDV (Md5) [11]. However, pathogenic MDV-1 DNA contained three units of a tandem direct repeat, each with 132 bp in the terminal and internal repeats, of the unique L fragment of MDV-1 DNA [6], while the presence of two units of tandem direct repeat in MDV-1 DNA fragment *BamHI* was reported by Becker et al. [2]. Presence of virulent/very virulent MDV serotype 1 strains were also reported in the Indian poultry flocks [7]. The sequencing of two field samples allowed to establish that the field virus contains two copies of 132 bp repeats and it is the very characteristics of pathogenic MDV and together with other supportive evidence i.e. presence of lesions characteristics of MD and history of mortality and vaccination (i.e. HVT and/or HVT + SB-1) indicated that the recent outbreaks of MD were due to very virulent strain of MD. The present study gives an insight into nature of the MDV circulating in the field based on its genetic characters.

Thus unifying the overall results of the present study it appears that primer pair *Bam*H1/*Bam*H2 amplified a 434 bp (two 132 bp repeats) fragment, in 32 out of 34 clinical samples indicate the presence of virulent oncogenic MDV in the field. The vaccine strain or non-pathogenic strains have increased no of 132 bp repeats. As all the commercial farms had adopted the bivalent vaccination protocol the recent outbreaks could be due to a very virulent strain of MDV-1. This finding points towards the increase in the virulence of the field virus involved in present outbreaks. i.e. very virulent MDV or very virulent plus MDV. PCR was found very satisfactory in detecting the presence of MDV either in feather follicle or in tissue samples. Sequencing study has proved beyond doubt that the two representative samples contained two 132 bp repeats indicating the virulent nature of the field virus.

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