VETERINARSKI ARHIV 80 (5), 683-692, 2010

Characterization of the ICP4 gene in pathogenic Marek's disease virus of poultry in Gujarat, India, using PCR and sequencing

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KALYANI, I. H., M. M. TAJPARA, M. K. JHALA, B. B. BHANDERI, J. B. NAYAK, J. H. PUROHIT: Characterization of the ICP4 gene in pathogenic Marek's disease virus of poultry in Gujarat, India, using PCR and sequencing. Vet. arhiv 80, 683-692, 2010.

ABSTRACT

A total of 34 clinical samples were collected for detection of Marek's disease virus (MDV) by polymerase chain reaction assays using primers M1.1/M1.8 to amplify a region of the ICP4 gene in layer birds of poultry. Primer set M1.1/M1.8 amplified a 318 bp product as against the expected 247 bp product in 30 samples out of 34 samples tested. To confirm the result, this primer was subjected to NCBI BLAST, and it was found that the primer specific segment of 318 bp does exist in the published sequence of Md5 and Md11BAC. The PCR product was sequenced and resulted in 273bp by direct sequencing. The sequence was analysed using NCBI blast and Clustal W with the published sequence of Gallid herpes virus-2 giving a matching score of 97, 96 and 90% indicating a highly conserved region. This shows that the MDV is prevalent in Gujarat.

Key words: Marek's disease virus, ICP4 gene, polymerase chain reaction

Introduction

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

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ISSN 0372-5480
Printed in Croatia

of Fabricius, immunosuppression, and high mortality. MD commonly appears in 3 to 4 week old chickens and gradually builds to a peak between 12 to 30 weeks of age.

The presence of MD and/or MDV has been reported from different states in India, like Orissa, Andhra Pradesh, Arunachal Pradesh and Tripura (PRADHAN and NAYAK, 1973; REDDY et al., 1980; VERMA et al., 1989). In Gujarat, cases of MD were reported to exist from 1971 and the incidence was increasing, as reported by VARIA et al. (1972). They found that on each farm in the study, five to 60 percent birds were found ailing, showing various symptoms suggestive of MD. On an average, mortality ranged from five to 30 percent in the older age group and in the younger age group, rarely less than 15 percent, commonly 20 to 30 percent and as high as up to 60 percent. POSIA (2003), screened ten serum and ten feather follicle samples from each of the 20 selected commercial broiler farms in Gujarat, and found that overall seroprevalence of MD was 7.5 percent.

The technique for diagnosis of infection with MDV is isolation and identification of MDV from infected tissues. Virus isolation is usually by virus propagation in cell culture and identification/quantification by cytopathic changes (plaque formation) or identification of the infected cells by immunostaining (De LANEY et al., 1998). Polymerase chain reaction (PCR) has emerged in recent years as an additional diagnostic tool, offering the advantages of serotype specificity (De LANEY et al., 1998) and the ability to differentiate between the vaccinal and wild strain of MDV serotype-1 (BECKER et al., 1992; SILVA, 1992; HANDBERG et al., 2001).

The MDV ICP4 homolog gene maps to the BamHI-A fragment, lying within the inverted repeat flanking the unique short region of the MDV genome (CANTELLO et al., 1994). The predicted coding region is 4,245 nucleotides long, and the predicted protein has an overall structure similar to those of other ICP4-related proteins in that two distinct regions of the protein are highly conserved. Several potential transcriptional regulatory sites, including ICP4 autoregulatory sites, octamer motifs, and potential cap sites, are present upstream and downstream of the predicted translational start site. A family of latency-related transcripts that is abundant in lymphoblastoid cells has been identified, and these transcripts are complementary to and overlap the predicted start site of translation of the MDV ICP4 homolog (CANTELLO et al., 1994; McKIE et al., 1995). The patterns of expression of these RNAs suggest that they are involved in the molecular switches important for turning off MDV replication when the virus resides in a non-permissive environment and needs to assume the latent state. In addition, stable transfection of a lymphoblastoid cell line with the ICP4 homolog gene expressed from a constitutive promoter results in dramatic up-regulation of pp38 gene expression (PRATT et al., 1994).

ROSS et al. (1997) described that the MDV EcoRI-Q gene (meq) and a small RNA antisense to ICP4 were abundantly expressed in CD4 cells and cells carrying a novel lymphoid marker, AV 37, in MD lymphomas. Northern blot analysis at lymphocyte

fractions, purified by immuno affinity, showed that CD4 and AV 37 fractions from lymphomas expressed meq and small RNA antigen antisense to ICP4 (SAR).

TULMAN et al. (2000) presented the first complete genomic sequence, with analysis of a very virulent strain of MDV serotype-1 Md5. The genome is 1,77,874 bp and was predicted to encode 103 proteins. MDV1 is a coline with the prototypic herpes simplex virus type 1 (HSV-1) within the unique long (UL) region, and it is most similar at the amino acid level to MDV2, herpesvirus in turkeys (HVT), and non-avian herpesviruses, equine herpesviruses 1 and 4. MDV1 encodes 55 HSY-1 UL regions homologous together with six additional UL proteins that are absent in non-avian herpesviruses. The unique short (US) region is colinear with and has greater than 99% nucleotide identity to that of the MDV strain GA. However, an extra nucleotide sequence at the Md5 US/short terminal repeat boundary results in a shorter US region and the presence of a second gene (encoding MDV 097) similar to SORF2 gene Md5, like HVT, encodes an ICP4 homologous that contains a 900-amino acid-amino-terminal extension not found in other herpesviruses. Md5 contains only two copies of the 132 bp repeat, which has previously been associated with viral attenuation and loss of oncogenicity.

The ICP4 gene belongs to a major category of genes in MDV, classified or grouped under genes homologous with alphaherpesviruses. This is a broad category of genes further divided into immediate early, early and late genes, which are, with few exceptions, important for virus replication. The primer to amplify ICP4 fragment of MDV-1 was designed as per the sequence published by HANDBERG et al. (2001).

Changes in MDV and MD are numerous and are brought about by many intercurrent forces. Understanding and anticipating these changes are fundamental to achieve further progress in the poultry industry. The technology of nucleic acid amplification is now widely applied in the diagnosis of poultry diseases. MDV infection has been known to occur in this state and country for the past 35 years or more, but work involving to detection of MDV by PCR and other aspects like genetic characterization by DNA sequencing has not been done. Keeping these facts in view, the present study was undertaken for the characterization of the ICP4 gene in MDV from clinical samples of chicken in layer birds by PCR and genetic characterization of the field MDV by sequencing the PCR targeted ICP4 gene segment, from the selected areas of Gujarat state, India.

Materials and methods

Samples. A total of 34 clinical samples for MDV detection by PCR were collected from 27 layer birds. A minimum of five feathers were collected from each 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 the 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. The birds were vaccinated with HVT + serotypes 2 virus.

Reference strains. One Lyophilized freeze-dried live HVT vaccine (HVT-126) obtained from the Division of Standardization, IVRI, Izatnagar, U.P., India and three commercially available vaccine strains, namely Cell free HVT vaccine, Cell associated HVT vaccine and Cell associated MDV-2 (serotype-2 MDV) vaccine, were obtained from Ventri Biologicals Ltd., Pune, India.

DNA extraction. Extraction of DNA from feather tips was carried out as per the procedure described by HANDBERG et al. (2001). DNeasy Tissue Kit (Catalog no. 69504, QIAGEN Pvt. Ltd) was used for extraction of DNA from tissue samples and vaccines.

PCR. The primers to amplify ICP4 fragment of MDV-1 were designed as per the sequence published by HANDBERG et al. (2001). PCR was performed using the forward primer M1.1 5' GGATCGCCCACCACGATTACTACC 3' and reverse primer M1.8 5' ACTGCCTCACACAACCTCATCTCC 3', targeting a nucleotide sequence in ICP4 gene (unique for MDV-1). Primer set M1.1/M1.8 amplified a 318 bp product as against expected 247 bp product in 30 samples. To confirm the results, these primers were subjected to NCBI BLAST, and it was found that the primer specific segment of 318 bp does exist in the published sequence of Md5 and Md11BAC. The primer for the targeting gene was synthesized from MWG, Bangalore, India. 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 $MgCl_2$, 200 µM of the four dNTPs, 10 pmol of each primer, and 1U Taq DNA polymerase (Sigma Aldrich, USA). The PCR tubes with all the components were transferred to a thermal cycler (Eppendorf Master Cycler, Germany). The PCR protocol used for the primer was initial denaturation at 94 °C for 1min, followed by 31 cycles of denaturation at 94 °C for 1min, annealing 55 °C for 10s and extension 72 °C for 1min, and final extension at 72 °C for 10 min.

Sequencing of M1.1/M1.8 primer amplified PCR product.

Materials. (a) One sample was selected from the PCR product of M1.1/M1.8 amplified a 318 bp. (b) BigDye® Terminatorv1.1Cycle Sequencing Kit with following components.

i) Ready reaction premix

ii) Big dye sequencing buffer (5X) iii) pGEM®--3Zf(+) double stranded control DNA template (c) Performance optimized polymer (POP-6™) (Part No. 402837 Applied Biosystems, USA) (d) Sequencing primer BamHI forward (Synthesized by Sigma Aldrich, USA) (e) Ethanol, (f) EDTA 125 mM (g) Sodium acetate (3M) (h) 70% Ethanol (i) Formamide (Sigma Aldrich, USA, Catalog No. F-9037).

Methods. Cycle sequencing was performed following the instructions supplied along with the 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. The composition of the reaction mixture for cycle sequencing contained 4 µL of 2.5X ready reaction premix (final concentration 0.5X), 2 µL of 5X big dye sequencing buffer (final concentration 1X), 1 µL forward primer BamHI (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 tubes with all the components were transferred to a thermal cycler. The cycling protocol for sequencing reaction was designed for 25 cycles, with initial denaturation at 94 °C for 5min, denaturation at 94 °C for 30sec, annealing 55 °C for 10sec, extension 72 °C for 4min and the thermal ramp rate of 1 °C per second.

Purification of extension products. Before subjecting the extension products for electrophoresis through the POP-6™ polymer filled capillary of the 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 completely by ethanol precipitation in the 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 minutes at room temperature, the precipitated DNA was pelleted by centrifuging at 11,000 rpm for 45 minutes at 4 °C, the pellet was washed with 70 µL of 70% ethanol by centrifuging at 11,000 rpm for 15 minutes at 4 °C. Finally the pellet was dried and re-suspended in 20 µL formamide.

Electrophoresis and data analysis. Electrophoresis and data analysis were carried out on the ABI PRISM® 310 Genetic Analyzer using appropriate Module, Basecaller, Dyeset/Primer and Matrix files.

Sequence analysis. The nucleic acid sequence obtained from the 273bp PCR products were aligned with known sequences of ICP4 of MDV genome available in Genbank®. The nucleotide sequences (excluding primer sequences) amplified by PCR were aligned using the Clustal W programme (THOMPSON, 1994).

Results

PCR. In the present study, 34 samples were tested for the presence of MDV-DNA using the primer M 1.1 / M 1.8. A total of 30 samples (15 feather and15 spleen) yielded a product of 318 bp instead of 247 bp product size, as per HANDBERG et al. (2001) (Fig. 1), while three HVT vaccines and one SB-1 (MDV-2) did not produce amplification.

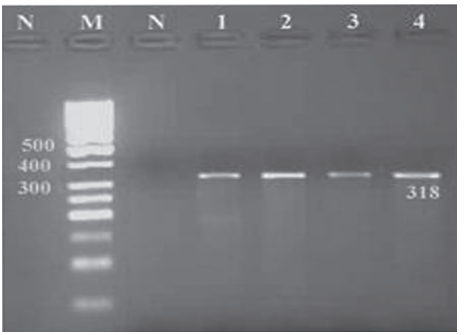

Fig. 1. Agarose gel electroforesis pattern of MDV1CP4 gene specific PCR products (Approximately 318bp) amplified with primer pair M 1.1/M 1.8. M: DNA molecular weight Markers: N: Negative control; 1-4: Field samples

Sequencing. A PCR (318bp approx) product of the above field sample was selected, purified and sequenced directly using the sequencing protocol as described earlier. The sample resulted in 273 bp (MDVICP4) long sequences, the remaining length of base pairs did not show a high confidence level and hence were not used for further analysis in the study.

Sequence obtained. >MDVICP4

5'GAAGAATCAGCCCGCGGTCTGGGGGGCGACGGAGATGGTGACCTAGATGATG
AAGGTGAGGACAGGACGATTAGAGAAGATGATGACGGTGAGGAT
GATGAAGAGGAGGAAGATGAAGAGGATGGCGATCTGGAAACATGT
CCGACGTTCCGGTGAGTAGTATTGGATGGGGGATTCCGGGGAGGGGATATA
ATAATGTGCTCTTTTGACGGTGAAGTCTCAGGGCCGCGTGGTCTGGGG
GAACCTCTTGGAGATGAGGTTGGGTGAGGCCT 3'

Table 1. Nucleotide -nucleotide BLAST of primer unique to ICP4 gene of published MDV sequences

Accesion No.	Location	Length	Location of ICP4	Length (bp)
gb/AF14806.2	145079-145397	318	141467-148438	6971
	167651-167379	318	164315-171286	
gb/AY510475.1	157726-158044	318	154120-161085	6965
gb/AF243438.1	147410-147728	318	143804-150769	
	171034-170716	318	167675-174640	

Table 2. Nucleotide -nucleotide BLAST of primer unique to ICP4 gene of submitted MDV sequences

Seq A	Name of sequence	Length (nt)	Seq B	Name of Isolate/sequence	Length (nt)	Score
	MDVICP4	273	2	gi|49349068_145079-145397 Gallid herpesvirus 2, complete genome	319	97
1	MDVICP4	273	3	gi|41387571_157726-158044 Gallid herpesvirus 2 clone Md11BAC replication intermediate, complete genome	319	96
2	gi|49349068_145079-145397	319	3	gi|41387571_157726-158044 Gallid herpesvirus 2 clone Md11BAC replication intermediate, complete genome	319	99
1	MDVICP4 Reverse compliment	273	2	gi|49349068_167379-167651 Gallid herpesvirus 2 complete genome	643	90
1	MDVICP4 Reverse compliment	273	3	gi|10180697_170716-171034 Gallid herpesvirus 2 serotype 1 isolate Md5, complete genome	319	96
2	gi|49349068_167379-167651	643	3	gi|10180697_170716-171034 Gallid herpesvirus 2 serotype 1 isolate Md5, complete genome	319	85

Discussion

Marek's disease (MD) is a lymphoproliferative disorder of chicken characterized by oncogenic transformation of T cells that infiltrates lymphoid tissues, peripheral nerves and visceral organs, resulting in complex pathogenesis that usually leads to the death of the affected birds. In the present study, using the primer i.e. M 1.1/M 1.8, to our surprise, instead of 247 bp product size, which was expected from a positive sample and reported by HANDBERG et al. (2001), 30 samples yielded a product of 318 bp, i.e. a difference of 71 bp. To confirm our result, we used the help of NCBI facilities. Taking forward and reverse primer sequences, we performed a Nucleotide -Nucleotide blast and it was found that (Table 1) there was more chance of obtaining the PCR fragment of about 318 bp. From Table 1 it is evident that the targeted product of 318 bp is very likely and, further, they are located in the ICP4 region of MDV only. The most possible reason for this could be that one of the primers might be capable of annealing to more than one site on the DNA.

Considering the above fact, the PCR product amplified by a primer pair M 1.1/M 1.8 was selected for sequencing analyses to discover exactly whether the amplified fragment contained the ICP4 region. A PCR (318 bp approx) product of the above field sample was resulted in 273 bp (MDVICP4) long sequences. To confirm the result, the obtained sequence of 273 bp (MDVICP4) was subjected to NCBI BLAST and Clustal W. It was found that the obtained sequence matched the published sequence gi|49349068_145079-145397 - Gallid herpesvirus 2 complete genome and gi|41387571_157726-158044-Gallid herpesvirus 2 clone Md11BAC replication intermediate complete genome, 97 percent and 96 percent respectively, while the reverse compliment (because genome of MDV consists of a unique long region and a unique short region bound by inverted repeats) of the obtained sequence MDVICP4 is a match for the published sequence gi|10180697_170716-171034-Gallid herpesvirus 2 serotype 1 isolate Md5 complete genome and gi|49349068_167379-167651-Gallid herpesvirus 2 complete genome, 96 percent and 90 percent respectively (Table 2). Alignment of 273 bp sequences and reverse compliment of the obtained sequence of the sample with previously published sequences of MDV isolates revealed that the representative field isolate of the present study shared maximum homology, as expected, due to the highly conserved region.

Hence, the primer pair M 1.1 / M 1.8 could be used for detection of MDV. The obtained sequence of 273 bp (MDVICP4) matched 97 percent with gi|49349068_145079-145397, 96 percent with gi|41387571_157726-158044 and gi|10180697_170716-171034 and 90 percent with gi|49349068_167379-167651. The alignment of the 273 bp sequence of MDDVICP4 with MDV published sequences, indicated that the sample contained a genome (fragment) of MDV-1 only. The PCR product was specifically selected for sequencing study, because the part of the MDV genome which is amplified with this set of primer, only exists in MDV-1 and not in MDV-2 or MDV-3 and the results were as per expectations and in accordance with other published reports concerning the same.

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Received: 25 August 2009
Accepted: 9 July 2010

KALYANI, I. H., M. M. TAJPARA, M. K. JHALA, B. B. BHANDERI, J. B. NAYAK, J. H. PUROHIT: **Karakterizacija gena ICP4 patogenoga virusa Marekove bolesti dokazanoga u peradi u Gujaratu u Indiji lančanom reakcijom polimerazom i sekvenciranjem. Vet. arhiv 80, 683-692, 2010.**

SAŽETAK

Prikupljena su 34 uzorka kliničkoga materijala nesilica za dokaz virusa Marekove bolesti lančanom reakcijom polimerazom uporabom početnica M1.1/M1.8 za umnažanje područja gena ICP4. Uporabom seta početnica M1.1/M1.8 umnožen je proizvod od 318 bp u odnosu na očekivani od 247 bp u 30 od 34 pretražena uzorka. Za potvrdu rezultata početnica je bila analizirana pomoću programa NCBI BLAST, te je ustanovljeno da početnica za specifičan odsječak od 318 bp postoji u objavljenoj sekvenci Md5 i Md11BAC. Proizvod PCR-a bio je izravno sekvenciran te je ustanovljeno da sadrži 273 bp. Slijed je bio analiziran uporabom programa NCBI BLAST i Clustal W i uspoređen s objavljenim slijedom za kokošji herpesvirus 2 te je ustanovljena podudarnost od 97, 96 i 90% što upućuje na genski jako očuvano područje. To pokazuje da je virus Marekove bolesti proširen u Gujaratu.

Ključne riječi: virus Marekove bolesti, gen ICP4, lančana reakcija polimerazom