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Identification and Molecular Cloning of Marek's Disease Virus Type 3 Glycoprotein M and Glycoprotein K

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Abstract

Marek's disease (MD) is a lymphoproliferative disease of chicken caused by Marek's disease virus (MDV), a cell-associated alpha-herpesvirus characterized by oncogenic transformation of T-cells that infiltrate lymphoid tissues, peripheral nerves and visceral organs. It is probably the most commonly occurring neoplasm of any animal population and until recently it caused extensive economic loss. MD has been effectively controlled over thirty years by vaccination using non-pathogenic, genetically and antigenically related serotypes (MDV-2 and MDV-3) or MDV-1 attenuated strains but despite vaccination more and more pathogenic MDV strains have evolved in recent years. The constant evolution of MDV has forced the development of new vaccines or strategies for vaccine production that control the emergence of more virulent strains. Hence, the ultimate aim of this study is to investigate the potential use of glycoprotein K and glycoprotein M of MDV-3 strain FC126 as immunogens against Marek's disease virus.

To address this issue genes of glycoprotein M and Glycoprotein K of MDV-3 strain FC126 were isolated by PCR amplification mediated methods using gene specific primers. The genes were cloned in *E.coli* using the pGEM-T Easy Vector. The cloned genes were sequenced and compared with related gene sequences from the GenBank. The sequence data was found to be identical with the data from NCBI GenBank. Expression patterns of glycoprotein M and K genes can help in developing a recombinant vaccine against the Marek's disease.

Keywords: MDV, Marek's disease, Tegument proteins, Glycoprotein, Recombinant vaccine.

Introduction

Marek's disease virus (MDV) is a highly oncogenic, cell-associated avian alpha-herpesvirus that causes oncogenic transformation of T-cells which may induce T-cell lymphoma in poultry. MDV is a member of the genus *Mardivirus* within the *Alpha-herpesvirinae* subfamily in the family *Herpesviridae*[1,2]. Based on the virulence and antigenic properties, MDV has been classified into three serotypes; MDV1, MDV2 and MDV3/HVT[3,4]. Among these, MDV-1 is highly infectious that causes cytolytic infection of both T and B-cells and may induce T-cell lymphoma in chicken[5]. MDV-2 consists of naturally occurring non-pathogenic MDV strains. MDV-3 or herpesvirus of turkey (HVT) is also apathogenic, originally isolated from turkeys[5].

Recent nomenclature reclassified these serotypes as gallid herpesvirus 2 (GHV-2), gallid herpesvirus 3 (GHV-3), and meleagrid herpesvirus 1 (MeHV-1), respectively[6]. Marek's disease virus (MDV) and closely related herpes virus of turkey (HVT) have been assigned primarily because of their tropism for lymphocytes[7]. HVT and MDV-2 have been used as a Marek's disease vaccine since early 1970s[3,4,8,9].

In the commercial poultry operations, Marek's disease has been controlled since the early 1970s by vaccination with live, non-oncogenic strains of MDV and/or a related virus, herpesvirus of turkey (HVT) [10]. Despite vaccination more and more pathogenic MDV strains have evolved in recent years[11]. These strains are classified as so-called virulent (v), very virulent (vv), or very virulent plus (vv+) strains[12,13]. The constant evolution of MDV has forced the development of new vaccines or vaccine strategies that control the emergence of more virulent strains[14].

The role played by MDV tegument and envelope (glyco) proteins in virus attachment, entry or in virus maturation and egress have remained elusive to a large extent, but it is known that the numbers of MDV structural proteins are the key players in all these processes[15]. Envelope glycoproteins represent major antigens recognized by the immune system of the infected virus. They have also been found to be of prominent importance for the infectivity of the free virions as well as for the capability of the virus to spread directly between infected and non-infected cells[16]. Marek's disease can successfully be prevented by vaccination with the non-pathogenic, genetically and antigenically related viruses (MDV-2 and MDV-3/HVT), or MDV-1 attenuated strains [13,17,18]. Hence the aim of this study was to clone glycoprotein K and M genes of HVT in *E.coli* which may be used for further expression in a eukaryotic system to produce recombinant vaccine.

Materials and Methods

Viral Inoculation and Harvesting (Propagation)

A vaccine strain (FC126) of HVT procured from Punjab Agricultural University (India) was used for propagation of the virus. Specific pathogen-free embryonated chicken eggs were collected 11days after fertilization and viral suspension was

inoculated into chorioallantoic membrane (CAM). The eggs were incubated at 37ºC for three days then CAMs were isolated on 3rd day aseptically and cleaned using sterile saline. Pock lesions were homogenized to a uniform suspension with Phosphate buffered saline (PBS). Homogenate was passed through a bacterial filter, and then filtered particles were resuspended in PBS and centrifuged for 10 minutes at 10000 rpm. Supernatant containing virus was collected and stored at -20ºC for further experiments.

Isolation of Viral DNA

Viral DNA was extracted as per the standard protocol[19] using Proteinase K and Phenol- chloroform extraction and purification method. The purified DNA pellet was dissolved in minimum volume of TE buffer and stored at -20 ºC until further use. The quality of the DNA was checked by 0.7% agarose gel stained with Ethidium bromide.

PCR Amplification of Glycoprotein Genes

The isolated DNA was subjected to amplification by PCR using gene specific primers which were designed based on the genomic sequence of HVT obtained from NCBI database. The PCR reaction mix consisted of 4 µl of $MgCl_2$ (25 mM), 3 µl of DNA template (20 ng/µl), 2.5 µl of 1×Taq buffer, 1 µl of dNTPs (10 mM), 0.6 µl of each primer (0.2 mM), 0.5 µl of Taq DNA polymerase (3 u/µl) and distilled water to make final volume of 25 µl. Amplification was performed in a Palm Thermal Cycler (Consort, USA) with the following amplification conditions: initial denaturation for 3 minutes at 94ºC, followed by 35 cycles consisting of denaturation at 94ºC for 1 minute, annealing at 55ºC for 2 minutes, and extension at 72ºC for 3 minutes and the reactions were held at 72ºC for 10 minutes as a final extension. The amplified PCR product was checked on 1.6% agarose gel.

Glycoprotein Gene Cloning

The amplified PCR products were ligated to pGEM-T Easy Vector (Fig.1) as per manufacturer's instructions. The ligated mixture was used for transformation of *E.coli* cells (JM109 strain). The transformants were screened on LB agar plates containing ampicillin (100 µg/ml) and IPTG (0.5 mM) and X-Gal (80 µg/ml). Plates were incubated overnight at 37°C and only white colonies appeared on LB agar were selected for further studies. These transformed colonies were checked further for the presence of DNA insert by isolating the plasmid and digesting with *Eco*RI. Glycoprotein genes thus cloned were sequenced by dideoxy chain termination method and was compared with similar sequences in the NCBI database.

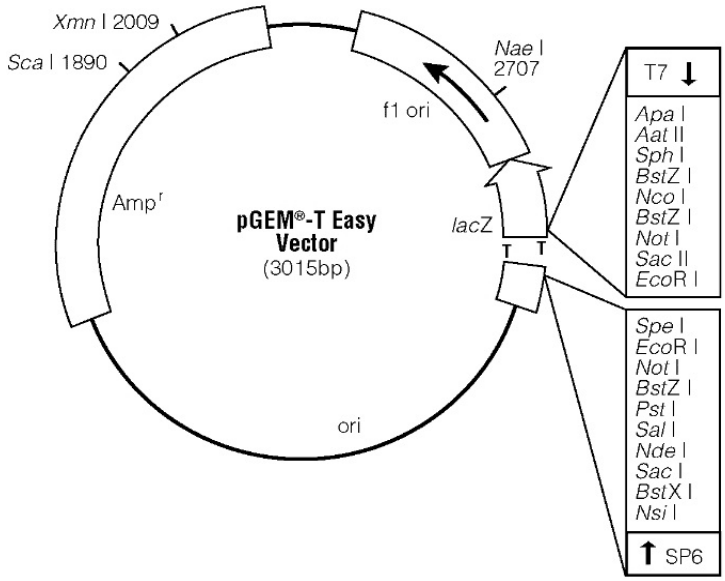

Figure 1: pGEM-T Easy Vector circle map.

Results

Isolation of Viral DNA

The purity and size of isolated viral DNA were checked on 0.7% agarose gel along with molecular markers. A single band was noted (Fig.2). The extracted viral DNA was used as template DNA for amplification of glycoprotein M and K genes.

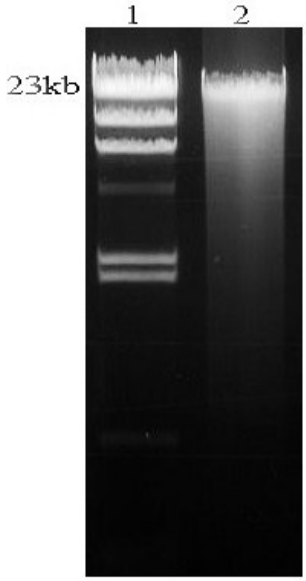

Figure 2: Genomic DNA isolated from Marek's disease virus type 3. Lane 1, Hind III-digested λ DNA; Lane 2, Genomic DNA isolated from lyophilized sample.

PCR Amplification of Glycoprotein Genes – gK and gM

The glycoprotein M and K genes were amplified using gene specific primers. Initial standardization by gradient-PCR facilitated the specific amplification as observed by high intense band. The optimum annealing temperature was found to be 55ºC. The amplification product was analyzed on 1.6% agarose gel and each single band approximately of 1.1 kb and 1.3 kb size were observed for gK and gM respectively (Fig.3).

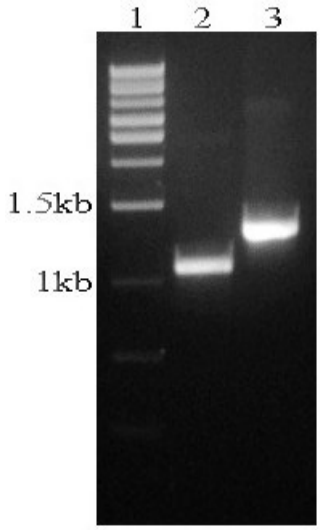

Figure 3: Amplified glycoprotein K and M genomic DNA units of Marek's disease virus (MDV). Lane 1, 1 kb DNA ladder (AXYGEN); Lane 2, Glycoprotein K genomic DNA with approximately 1.1kb; Lane 3, Glycoprotein M genomic DNA with approximately 1.3 kb.

Transformation and Cloning of Glycoprotein Genes

Amplified PCR products were purified from low melting agarose gel as per the standard protocol[19]. The purified amplification products ligated to pGEM-T Easy Vector (Promega, USA) when monitored on 1% agarose gel confirmed the successful ligation on observing the ligated product as a single band. The transformation mixture containing the recombinant plasmids on transformation of *E.coli* strain JM109 when plated on an agar plate supplemented with Ampicillin (100µg/ml) and X-Gal (80 µg/ml) and IPTG (0.5 mM) after overnight incubation, the transformants that contained recombinant plasmids appeared creamy-white colonies while non-recombinant transformants appeared as bluish. The percentage of recombination was ascertained to be much higher as there was very high number of recombinant colonies compared to the non-recombinant blue colonies.

Isolation of Plasmid

The isolated plasmids from white colonies were found to be migrating slower than the control vector, indicating that these are positive for the presence of DNA inserts. The presence of the correct DNA insert form was confirmed by restriction digestion analysis of the recombinant plasmids isolated from the white colonies and the standard pGEM-T Easy Vector using *Eco*RI. Two bands-insert and the plasmid, of expected sizes were observed in both the samples which confirmed that plasmids were recombinants that contained the correct type of DNA insert (Fig. 4).

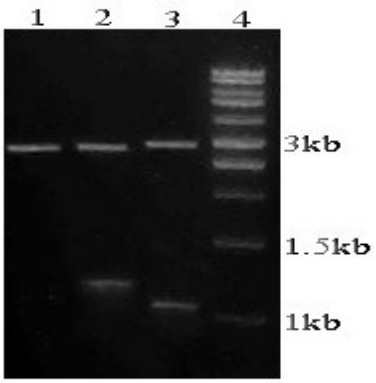

Figure 4: Restriction enzyme digestion of recombinant plasmids. Lane1, linerized pGEM-T Easy Vector; Lane 2-3, *Eco*RI- digested pGEM-T Easy Vector with glycoprotein M, K genes respectively; Lane 4, 1 kb DNA ladder (AXYGEN).

Sequence Analysis

The purified plasmids when sequenced from forward direction using the sequencing facility of Axygen Scientific (P) Ltd (New Delhi, India), sequence of 1049 bp for gK and 1271 bp for gM could be read from the ladder sequences which were found to be perfectly matched with the sequence data obtained from NCBI.

Table 1: Sequences of the oligonucleotide primers used in the study. Primers were designed based on NCBI sequence database.

Primers	Sequence (5´-3´)
gK Forward	CGATTCGAAATGTCGTACAGACCAAC
gK Reverse	GACTCGGTTATGGCAAATATGCACGT
gM Forward	CGATACGTCATGGATGGCCAAACAAA
gM Reverse	CCAGATAATGTTAATCATCCCACTC

Discussion

Marek's disease is a very contagious neoplastic disease in chicken which was described nearly a century ago by Professor Marek. Poultry industry sustains enormous loss due to Marek's disease each year. It has been controlled soon after isolation of MDV (1970s) by vaccination using non-pathogenic, genetically and antigenically related serotypes (MDV-2 and MDV-3) or MDV-1 attenuated strains. Despite vaccination, more pathogenic strains have also been emerging. Hence the goal of researchers in this area has always been making recombinant vaccine which can control the newly emerged pathogenic strains. Since the role of glycoproteins of MDV in virus attachment, entry, egress and virus maturation has been cleared, in the present study two of the glycoproteins (K and M) were used for making recombinant vaccine. As an initial step towards this goal, the virus was inoculated into CAM and propagated, then viral DNA was isolated and glycoprotein genes were amplified using PCR. In the next step amplified genes were cloned into *E.coli* using pGEM-T Easy Vector system. The plasmids were isolated from transformants and digested by restriction enzyme to confirm the presence of insert gene. Final expression steps of the recombianant vaccine are in progress.

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