

Molecular cloning and analysis of glycoprotein genes C, D and I of an isolated strain of Marek's Disease Virus Type-3

Smitha Sudhakar* & A. Jayakumaran Nair

*Department of Biotechnology, University of Kerala,
Thiruvananthapuram, Kerala.*

*smita006@gmail.

Abstract

Marek's disease is the most commonly occurring neoplasm of any animal population and until recently it has caused extensive economic loss globally. Marek's virus, an actively evolving virus is the causative agent. Now with the virus's genetic code in hand we could easily characterise the immunogenic proteins of the closest relative of the virus, the Herpes virus of Turkey's. The important glycoprotein genes gC, gD, and gI of an isolated strain of Herpes virus of Turkey's were amplified, cloned in pGEM T-Easy vector and sequenced as an initial step in developing a recombinant vaccine against Marek's Disease. The recombinant genes were analysed and sequence data confirmed that the isolated strain was HVT (FC126), a vaccine strain

Keywords: Marek's Disease, Oncogenic, Glycoproteins, Herpesvirus, Poultry

1. Introduction

Marek's disease (MD) is a lymphoproliferative disease of chicken caused by Marek's disease virus (MDV), a cell associated herpesvirus, characterised by oncogenic transformation of T cells that infiltrate lymphoid tissues, peripheral nerves and visceral organs. MDV is classified as oncogenic serotype-1, non-oncogenic serotype-2 and serotype-3 alpha herpes virus that share antigenic similarity with each other and is wide spread in almost all poultry populations (Bulow & Biggs, 1975). MDV-1 is subdivided into pathotypes, which are referred to as mild (m) MDV, virulent (v) MDV and very virulent (vv) MDV, very virulent plus (vv+) MDV (Witter, 1997).

MD is highly transmittable among chickens and spreads through the gulp of air of infectious dander (Beasley *et al.*, 1970). Paralysis, skin leukosis, depression and death are the common clinical symptoms (Smith *et al.*, 1974; Witter, 1971; Ficken *et al.*, 1991). Neural and visceral lymphomas, bursa and thymus atrophication, splenomegaly and ultimately death will result. Since MDV was identified as the etiologic agent in the late 1960s (Churchill & Payne, 1969; Churchill & Biggs, 1967; Solomon, *et al.*, 1968; Witter *et al.*, 1970). MD has been controlled to a greater extent through vaccination using MDV-1 attenuated strains (Churchill & Payne, 1969), herpevirus of turkeys (HVT) (Witter *et al.*, 1970) and an apathogenic, MDV-2 strain isolated from chickens (Schat, 1981). In recent times, MD has been controlled principally with HVT and bivalent vaccines comprised of MDV-2 strain (SB-1) and HVT. As poultry production intensifies simultaneous with the advent of new vaccines coupled with evolutionary pressure there has been tremendous rise in the virulence of MDV field strains (Witter, 1997).

The glycoproteins of herpes virus mediate fundamental aspects of infection such as attachment, membrane fusion, penetration, transport of virion components, virion assembly, egress and cell-to-cell spread *etc.*, apart from their role as the virion surface components (Lubinski *et al.*, 1998). HVT glycoproteins represent potent immunogens against MDV since they are antigenically related strains (Witter, 1998) and hence, several herpesvirus glycoproteins like gC, gD and gI have evolved immunoevasive functions (Rajcani & Vojvodova, 1998).

1.1 Objectives

The objective of the study was to identify and characterize the above mentioned glycoproteins of an isolated HVT strain. These glycoproteins were reported to have immunogenic properties that can be utilized to develop recombinant vaccines against MDV infection. This project was initiated to analyze the immunological activity of recombinant HVT glycoproteins against MDV1. To address this, the major surface glycoprotein genes gC, gD and gI of that particular strain of HVT were amplified, cloned and sequenced.

2. Materials and Methods

2.1 Viruses and cells.

Primary chick embryo fibroblasts (CEF) were maintained in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum. The CEF were infected 4 hours after seeding. 0.5 plaque-forming units of HVT per cell were used as inoculum. The cultures were then incubated for 72 hours, when a cytopathic effect was apparent to 80% of the cells, harvesting was done as per the protocol described by Wyn-Jones & Kaaden (1979).

2.2 DNA Extraction

The infected CEF cells were washed with PBS and 4ml of lysis buffer at pH 8.0 (0.5%SDS, 100 mM NaCl, 1 mM EDTA, 200 µg/ml proteinase K in 10 mM Tris). The lysate was incubated at 37° C for 4 hours and an equal volume of TE-saturated phenol was added. The lysate was mixed by inverting the tube gently for 15 min and centrifuged at 1500xg for 5 min. The aqueous layer was transferred to a new tube and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The tube was mixed gently and centrifuged at 1500xg for 5 min. The aqueous layer was mixed with two volumes of 100% ethanol and mixed gently in a new tube. Then the tube was centrifuged at 1500xg for 5 min. The precipitated DNA were rinsed with 70% ethanol, dried and suspended with 0.1 ml of TE (Sambrook *et.al.*, 1989). The DNA was run on 0.6% agarose gel and visualized (Figure 1).

2.3 PCR conditions and amplification

Total DNA preparations from HVT infected CEF were used as templates for PCR amplification. Oligonucleotide primers were designed for PCR amplification that spans the translational start and stop codons for the 3 glycoproteins gC, gD and gI of HVT in general (Table 1). DNA (500 ng) was mixed with 50µM of each of dNTPs and 2 pM of each of the primers in a final volume of 45 microlitres. The mixture was overlaid with mineral oil (Sigma) and heated to 98°C for 10 min in a MJ Research Programmable Thermal Controller. The reaction was cooled to 55°C for 10 min. During this time 5 microlitre of PCR buffer (500 mM KCl, 100 mM Tris-HCl, 15 mM MgCl₂, 1% Triton X-100) and 2.5 units of Taq polymerase (Promega) were added. A negative control was included from which, DNA was omitted. The PCR products were run on a 1.6% agarose gel in Tris Borate/EDTA buffer (Sambrook *et.al.*, 1989). The gels were stained with ethidium bromide to identify the bands and their sizes.

Table 1. Primers for amplification of glycoproteins

g ^C Forward	5' CTC GAG AAA AGA ATG ATT ATT GTC ACC ACT TCG 3'
g ^C Reverse	5' GCG GCC GCT CAT AGC CTG GTA TAC ACA TAC CGG C 3'
g ^D Forward	5 CTC GAG AAA AGA ATG CTT ATG ACT CCT ACA ATG 3'
g ^D Reverse	3'GCG GCC GCC TAT ACA ATT TCA TCA TCC GTC TC 5'
g ^I Forward	5' CTC GAG AAA AGA ATG GTT TCC AAC ATG CGC G 3'
g ^I Reverse	3' GCG GCC GCT TAA TTC CGC CCC GGT AGG TAA AAG 5'

2.4 Transformation and Cloning of amplicons in pGEM T-Easy vector

The amplified glycoprotein genes were purified using Axyprep PCR purification kit and were cloned using pGEM T-Easy vector system (Promega Inc.).

2.4.1 Ligation Using 2X Rapid Ligation Buffer

The pGEM T-Easy vector was briefly centrifuged. Set up ligation reactions as described in Table 2. Vortex the 2X Rapid Ligation buffer vigorously and mix the contents by pipetting. Incubate the reactions overnight at 4°C for the maximum number of transformants.

Table 2. *Ingredients for ligation mixture*

Reagents	Standard reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4DNA Ligase	5µl	5µl	5µl
pGEM-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	2µl	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
Deionized water to a final volume	10µl	10µl	10µl

2.4.2 Transformation of JM109 Competent Cells and cloning

For each ligation reaction two LB, Ampicillin, IPTG, X-Gal plates were prepared. The ligation reactions were centrifuged, 2µl of each ligation reaction were added to a sterile 1.5ml tube on ice. Transfer 50µl of JM109 cells in to each tube and flick the tubes to mix and place them on ice for 20 minutes. Heat-shock the cells for 45-50 seconds in a water bath at exactly 42°C and return the tubes to ice for 2 minutes. Add 950µl room temperature SOC medium to the tubes containing cells transformed with ligation reactions and incubate for 1.5 hours at 37°C with shaking (~150rpm). 100µl of each transformation culture were plated on to LB/Ampicillin /IPTG /X-Gal plates. The plates were incubated overnight at 37°C.

2.5 Restriction Digestion Analysis of Plasmids

The presence of the correct DNA insert form was confirmed by restriction digestion analysis of the recombinant plasmids using PstI and EcoRI separately. After checking the linearized plasmids by PstI on 0.8% agarose gel it was found that the isolated plasmids from white colonies were migrating slower than the control vector indicating that these are positive for the presence of DNA inserts. Investigation for presence of insert DNA was also done by restriction digestion of plasmids using EcoRI to release the inserts. Two bands-insert and the plasmid of expected size were observed in the gel.

2.6 DNA sequence analysis

The DNA sequence analysis was performed with the cloned glycoprotein genes using vector-based forward and reverse primers with the DNA Sequencing Facility ABI 310. The sequence data was compared and aligned using NCBI database and nBLAST.

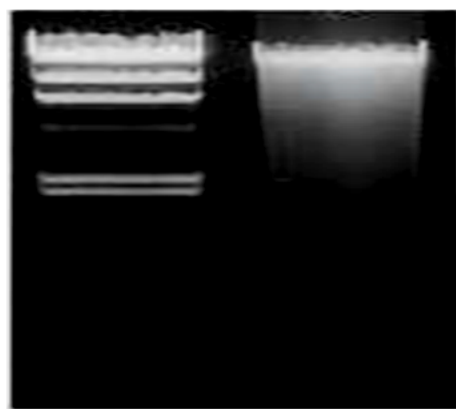
3. Results

3.1 Amplification and cloning of glycoprotein genes

The amplified PCR products were run on 1.6% agarose using 1X TBE buffer and compared with a DNA ladder (Figure 2). The amplicons were visualized using a UV transilluminator and found to be of the expected molecular weight- gC (~1470kb), gD (~1115kb) and gI (~1070kb). The amplicons were purified using Axygen PCR clean-up kit. Since the amplicons lacked restriction sites they were subjected to ligation under standard ligation conditions with pGEM T-Easy vector and the products run on 1% agarose and were seen as a single band. The ligated purified products were transformed to JM109 cells and plated on ampicillin plates containing X-gal and IPTG respectively. After overnight incubation, the recombinant transformants were seen as creamy-white pin-point colonies. Non-transformants were identified as bluish in appearance.

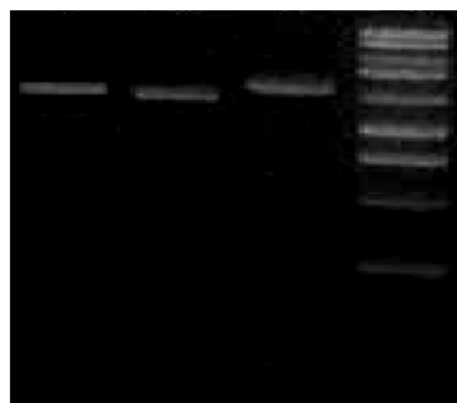
3.2 Recombinant plasmid isolation, amplification and restriction digestion

The number of recombinants was found to be much higher than that of the non-recombinants. Out of the numerous colonies, 6 of them were selected based on their color and appearance for enrichment and subjected to plasmid isolation. The isolated plasmids were run on 0.7% agarose. The plasmids were also subjected to EcoRI digestion which could release the cloned insert to reconfirm the transformation (Figure 3). The plasmids were amplified with respective primers and products were run on 1.6% agarose and compared with DNA ladder. The products were found to be of expected sizes confirming the success of cloning (Figure 4)

Fig.1. HVT DNA isolation

**Hind III λ
DNA digest**

HVT DNA

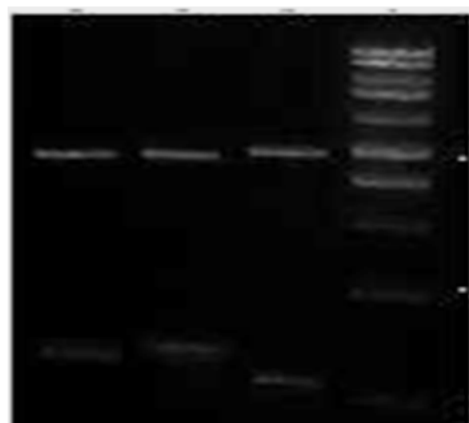
Fig.2. PCR products

gD

gI

gC

**1kb DNA
ladder**

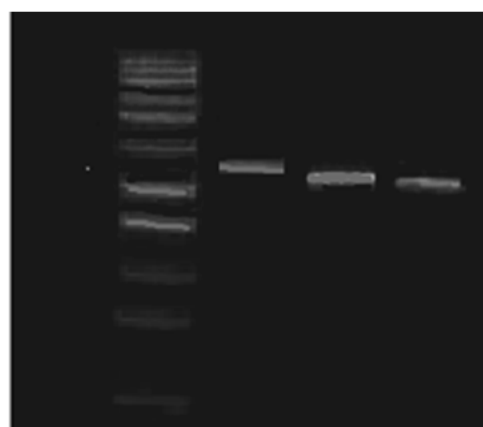
Fig.3. Recombinant plasmid digestion

gC

gI

gD

**1kbDNA
ladder**

Fig.4. PCR amplification of the plasmids

**Negative
Control**

**1kb DNA
Ladder**

gC

gD

gI

3.3 Sequencing, comparison and alignment of the inserts

The vector with insert was sequenced and the data was compared with NCBI database. The compiling and alignment was done using Nblast. The sequence showed 98% similarity to that of the glycoproteins gC, gD and gI of HVT strain FC126.

4. Discussion

Marek's disease is a lymphoproliferative disorder of chicken characterized by oncogenic transformation of T cells that infiltrate lymphoid tissues, peripheral nerves and visceral organs, resulting in a complex pathogenesis that usually leads to death of the affected birds. The causative agent of MDV is serotyped as MDV-1, 2 and 3 (HVT). Here the major envelope glycoprotein genes C, D and I of an isolated strain of HVT were amplified and cloned with pGEM T-Easy vector. The number of non-recombinants outstands that of the recombinants usually, but here it is not so since the restriction sites for the vector and the genes were different so that their ends could not stick to themselves as 2 different restriction enzymes were used. The inserts were identified as glycoprotein genes gC, gD and gI of HVT strain FC126 by sequence alignment and comparison. These glycoproteins are of prime importance since they are responsible for the major viral replication processes. These glycoprotein genes can be expressed in a suitable vector for the mass production of these envelope glycoproteins, which play a major role in egression, host specificity, viral replication and immunity conference. These glycoproteins can act as potent immunogens or MDV neutralizing agents. Hence the scope of using of these glycoproteins singly or in combination may be exploited to develop new vaccines against MDV1 in future.

5. References

- 1• Beasley, J. N., Patterson, L. T and Mc Wade, D. H (1970). Transmission of Marek's disease poultry house dust and chicken dander. *American Journal of Veterinary Research*. 2:339-344.
- 2• Bulow, V. V., and Biggs, P. M. (1975). Differentiation between strains of Marek's disease virus and Turkey herpes virus by immunofluorescence assays. *Avian Pathology*. 4:133-146
- 3• Churchill, A. E, and Biggs, P. M (1967) Agent of Marek's Disease in Tissue Culture. *Nature*, 215: 528-530
- 4• Churchill.A.E and Payne.L.N (1969). Immunization against Marek's disease using a live attenuated virus. *Nature*, 221: 744-747
- 5• Ficken, M. D, Nasisse, M. P Boggan, G. D, Guy. J. and Wages. D. P., Witter, R.L, Rosenberger, J. K, and Nordgren, R. M (1991). Marek's disease virus isolates with unusual tropism and virulence for ocular tissues: clinical findings, challenge studies and pathological features. *Avian Pathology*. 20:461-474.
- 6• Lubinski, J. M, Wang,L , Soulika, A. M, Burger, R., Wetsel, R. A., Colten.H, Cohen. G. H., Eisenberg, R. J, Lambris. J. D and Friedman, H. M (1998). Herpes simplex virus type I glycoprotein gC mediates immune evasion. *Journal of Virology*. 72:8257-826.
- 7• Rajcani, J., Vojvodova, A (1998). The role of herpes simplex virus glycoproteins in the virus replication cycle *Acta Virologica*. 42, 2. 103-118,
- 8• Sambrook, J., E, Fritsch.F, and T. Maniatis (1989) *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 2:202-205.
- 9• Schat. K. A, Calnek B. W and Fabricant, J (1981). Influence of the bursa of fabricius on the pathogenesis of Marek's disease. *Infection and Immunity*. 31:199-207.
- 10• Smith, T. W, Albert. D. M., Robinson, N, Calnek. B W, Schwaec. O (1974). Ocular manifestations of Marek's disease. *Investigative Ophthalmology*. 13:586-592.
- 11• Solomon, J. J, Witter. R. L, Nazerian, K.and Burmester, B. R (1968). Studies on the etiology of Marek's disease. I. Propagation of the agent in cell culture. *Proceedings of Society of Experimental Biology Med*. 127:173-177
- 12• Witter, R. L., Nazerian. K., Purchasc, H. G., and Burgoyne,G.H (1970) Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. *American Journal of Veterinary Research*. 31:525-538.
- 13• Witter, R. L (1971). Marek's disease research-History and perspectives. *Poultry Science*. 50:333-342.
- 14• Witter, R.L (1997) Increased virulence of Marek's disease virus field isolates. *Avian Dis*. 41:149-163
- 15• Witter. R.L. (1998). Control Strategies for Marek's Disease: A Perspective for the Future , *Poultry Science* 77:1197-1203
- 16• Wyn-Jones, A. P. and Kaaden, O. R (1979). Induction of vims-neutralizing antibody by glycoproteins isolated from chicken cells infected with a herpes virus of turkeys. *Infection and Immunity*. 25:54-59