

Original Research Article

<https://doi.org/10.20546/ijcmas.2018.710.326>

Expression Profiling of Glycoprotein D Gene of Bovine Herpes Virus in Madindar by Bovine Kidney Cells via Real Time PCR

Asmita Singh* and Ajay Kumar

Animal Nutrition Division, ICAR- Indian Veterinary Research Institute,
Bareilly, UP, 243122, India

*Corresponding author

ABSTRACT

Bovine herpesvirus-1 (BHV-1) a member of the *Herpes viridae* family and *Alpha herpes viridae* subfamily genus *Varicello virus* is a most common viral pathogen found in bovine semen. The entry process of Alpha herpes viruses consist of two steps, initial virus attachment and subsequent virus penetration involving membrane fusion as in other alpha herpes viruses, BHV-1 glycoproteins are the major structural components of the viral envelope and virus-infected cell membranes. Glycoprotein D (gD), of BHV-1 is one of four major glycoproteins, namely gB, gC, gD, and gH that have been identified on the virus envelope and the plasma membranes of BHV-1-infected cells [14, 24]. Total of 73 open reading frames (ORFs) have been clearly identified which are homologous to genes found in other alpha herpes viruses. The BHV-1 genome encloses ten genes encoding the glycoproteins, among them, six genes are in the UL region, namely gK (UL 53), gC (UL 44), gB (UL 27), gH (UL 22), gM (UL 10), gL (UL 1) and four remaining genes are in the Us region, gG (US 4), gD (US 6), gI (US 7) and gE (US 8). In the present study, a time course expression profile analysis of gD (US 6), was carried out at 6hr, 12hr, 24hr of h post infection quantitative RT-PCR as well as Real Time PCR. Gapdh was used as reference control to normalize the expression levels. A significant decrease in gD expression at 3 h post infection (h.p.i) was observed followed by an increase in between 6 hr to 12 hr h.p.i. Expression of gC gene become static in between 12 to 24 hr. However, further studies need to be carried out to unfold the molecular mechanism of interaction between host and virus to devise efficient control strategies for this chaos in the virus infection.

Keywords

Glycoprotein D,
Expression,
Alpha herpes
viruses, Herpes
viridae

Article Info

Accepted:
20 September 2018
Available Online:
10 October 2018

Introduction

The dairy sector has played a very vital role in the economic development of India through 63 years of Independence. India now produces 102.9 million tons of milk (FAO, 2007), world's largest milk producing country. To this, buffaloes contribute 55% and cattle 45% of total milk production. With the introduction

of cross-breeding programs, these animals have become susceptible to many diseases like Infectious bovine rhino-tracheitis /infectious pustular vulvo-vaginitis (IBR/IPV), caused by Bovine Herpes Virus-1 (BHV-1). Bovine herpesvirus-1 (BHV-1) a member of the *Herpes viridae* family and *Alpha herpes viridae* subfamily genus *Varicello virus* is a most common viral pathogen found in bovine

semen. BHV-1 virion is enveloped containing an icosahedral capsid surrounded by tegument, replicates in the nucleus but the envelope is acquired while budding through nuclear membrane. It is an important pathogen of cattle causing two major clinical syndromes, namely known as Infectious Bovine Rhinotracheitis (IBR) and Infectious Pustular Vulvovaginitis (IPV) (Kit *et al.*, 1989; Wyler *et al.*, 1989; Tikoo *et al.*, 1995). A total of 73 open reading frames (ORFs) have been clearly identified which are homologous to genes found in other alpha herpes viruses. The BHV-1 genome encloses ten genes encoding the glycoproteins, among them, six genes are in the UL region, namely gK (UL 53), gC (UL 44), gB (UL 27), gH (UL 22), gM (UL 10), gL (UL 1) and four remaining genes are in the Us region, gG (US 4), gD (US 6), gI (US 7) and gE (US 8). BHV-1 infection of permissive cells is initiated first by the low affinity interaction between viral gB and/or gC to the cell surface structures like heparin sulfate sugar moieties (Li *et al.*, 1995). This is followed by the stable binding of BHV-1 gD to cellular specific receptor and subsequent virus penetration by fusion of the virion envelop with the plasma membrane (Liang *et al.*, 1989).

The entry process of alpha herpes viruses consists of two steps, initial virus attachment and subsequent virus penetration involving membrane fusion. Glycoprotein D (gD) of the BHV -1 is an essential envelope protein, and it has been previously documented that gD plays a significant part in the virus entry steps (Liang *et al.*, 1997). Glycoprotein D (gD) of BHV-1 has been shown to be an essential component of virions involved in virus entry (Babiuk *et al.*, 1996). gD expression in infected cells is also required for direct cell-to-cell spread (Schroder *et al.*, 1997).

The first interaction involves low affinity virus attachment between gB and /or gC to cell

surface structures like heparan sulfate sugar moieties. This is followed by the stable binding of BHV-1 gD to cellular specific receptors. After this high affinity interaction between gD and cell receptors, the subsequent virus penetration occurs by fusion of the virion envelope with plasma membrane. This crucial process requires at least the involvement of four glycoproteins gD, gB and the heterodimer formed by gH and gL (B. Muylkens *et al.*, 1995). Live but also inactivated BHV-1 induced PBMC apoptosis suggested that a structural component of BHV-1 has the capacity to activate the apoptotic process. Moreover, only the attachment of BHV-1 was able to induce programmed cell death. Glycoprotein D was shown to be involved in this BHV-1, induced apoptosis (Hanon *et al.*, 1996).

These glycoproteins, of which gB, gC, and gD have been identified as the major immunogens recognized by sera from infected cattle (Collins *et al.*, 1985, van Drunen Little- van Hurk and Babiuk, 1986; Marshal *et al.*, 1986). Glycoproteins gB and gD are essential for virus replication and are responsible for inducing neutralizing antibody responses in the host. Although gC is dispensable for replication and induces lower neutralizing antibody response, it is immunodominant and is believed to play a role in initial attachment (reviewed in Tikoo *et al.*, 1995) These properties make the three proteins excellent target antigens for detection and analysis of BHV-1 immune responses. All of these factors make it important to develop diagnostics and vaccines, unless they are based on molecular techniques

Understanding the role of glycoprotein D gene in interaction between the cell and virus at molecular level would greatly help us in formulating effective therapeutic strategies. Hence a study was conducted on the expression profiles of the gc gene (US6 region

of open reading frame) containing proteins glycoprotein D in BHV -1 infected MDBK cells.

Materials and Methods

MDBK culture

Madin - Darby Bovine Kidney (MDBK) cell line was kindly provided by NCCS Pune. This cell line was maintained in DMEM supplemented with 100U/ml penicillin and 100 g/ml streptomycin and 10% fetal calf serum in 25 sq cm flask. This cell was used for propagation of BHV-1.

Preparation of experimental culture plate

MDBK cells were observed under microscope for confluence and then medium was decanted and monolayer was washed with serum free medium. 1 ml trypsin-versene solution was added to the cells in 25 sq. cm flask and incubated at 37°C for 2 min. The excess trypsin-versene was discarded and flask was re-incubated till cells started coming out from the monolayer. The cells were completely taken off from monolayer by gently flushing with pipette and growth medium containing 10% FCS was added and cells were dispersed equally in 24 well plate. The 24 well plate was incubated at 37°C till monolayer was formed

Infection of virus in MDBK cell lines

MDBK cell lines were full grown in 25cm² flask and we prepare a six well plate. After completion of complete monolayer in each well infect these cells by BHV-1 viruses. Add 100µl of virus was infected into well and one with cell control. Virus was allowed to adsorb on the cells by incubating the flask at 37°C for 1 hr with intermittent shaking after every 10 min. After adsorption, inoculum was discarded and 5 ml maintenance medium containing 2% FBS added and cells were

incubated at 37°C. There was cytopathic effect (CPE) after 24hr. The virus infected cells were harvested at 6hr, 12hr, 24hr of h.p.i and preserved in 1ml of TRI-REAGENT (MRC) for isolation of total RNA. After completion of monolayer, the medium from plate was drained. The cell monolayer was washed once with maintenance medium and the cells were infected by adding 0.5 ml of virus stock.

Extraction of BHV-1 RNA from cell culture fluid

Total RNA from infected and control cell culture fluid at different time points were isolated using TRIzol reagent (Invitrogen, USA) based on manufacturers' instructions after 3hr, 6hr, 12hr, and 24hr of post infection. TRIzol reagent was used for lysis of monolayer in 25cm² flask. Around 1ml TRIzol reagent was added directly on monolayer and flushed with the help of pipette. Further, the following steps were carried out as follows: 200 µl of chloroform was added and tube was vigorously vortexed for 2 min and incubated at room temperature for 10 minutes. Mixture was centrifuged at 10,000 rpm for 10 minutes at 4°C for phase separation. Carefully, the upper aqueous phase was removed transferred into a new 1.5 ml DEPC treated micro-centrifuge tube. 500 µl of isopropanol for 1.0 ml TRIzol reagent was added to the aqueous supernatant and mixed by upside down and incubated at room temperature for 10 min and was centrifuged at 12,000 rpm for 10 min at 4°C for RNA precipitation. Supernatant was discarded and pellet was washed with 0.5 ml of 75% ethanol and centrifuged at 7,500 rpm for 10 min at 4°C. Finally, pellet was dried at 50°C for 10 min and re-suspended in 50 µl DEPC treated water.

cDNA synthesis

The total RNA was quantified by measuring at 260 280 nm in Nanodrop spectrophotometer.

The ratio of A260 and A280 was calculated to check the purity of extracted RNA and quality was checked on a 1 % agarose gel. Total RNA was treated with RNase free DNase 1 (Fermentas, USA) to remove DNA contamination. The concentration of RNA was determined by Nanodrop spectrophotometer so that in cDNA synthesis taken same amount of RNA having same concentration which is measure by Nanodrop in order to prevent use of internal control. First strand cDNA synthesis was carried out using 1 µg of DNase-treated total RNA as template. Reverse transcription was performed using Moloney leukemia virus reverse transcriptase (Fermentas, USA), Random hexamer primer (0.2 µg/ µl), 1× RT reaction buffer, dNTPs (10mM), 20 U of ribonuclease inhibitor and 40 units of reverse transcriptase in a final reaction volume of 20 µl. The reaction was carried as per the manufacturers' instructions.

Semi quantitative RT-PCR analysis

Semi quantitative RT-PCR was performed using 50µg cDNA as template. The primers FP: 5'CCCGATGCCGCGATACTACT3', RP: 5'CGCACCCGCTCTCGATCTTG -3' were used to amplify glycoprotein D gene to generate 236 bp fragments of gC gene of BHV-1.β actin was used as internal control and was amplified with the primers pair The thermocycling parameters consisted of an initial denaturation at 94°C for 4 min followed by 30cycles of 94°C for 30 s, 58°C for 45s and 72°C for 1 min. The final extension was done for 5 min at 72°C. Hold temperature was set at 4°C. The PCR products were run on 1.5% gels for further analysis. PCR products were quantified using Gene Tools Software.

Real time PCR analysis

Real-time PCR amplifications were carried out in Mx3000P spectrofluorometric thermal cycler operated by MxPro TMQPCR software

using SYBR Green (Fermentas, USA) chemistry. The same primer pairs used in Semi-quantitative RT-PCR were used to amplify both glycoprotein D gene and internal control β actin.

The thermal profile used for PCR amplification consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 58°C for 30sec. Melting curve analysis of amplification products were performed at the end of each PCR reaction to confirm that only one PCR product was amplified and detected.

To assess the specificity of amplified product, dissociation curve were generated at temperature between 55°C and 95°C and the results was expressed in threshold cycle (Ct value).

Comparative CT method was used to estimate the relative expression of the target mRNA. Briefly, ΔCT was calculated by subtracting CT value of internal control from target gene and then mean ΔCT was calculated from this normalized ΔCT value. ΔΔCT was calculated with respect to control by, subtracting mean ΔCT of control treatment from mean ΔCT of the target gene. Fold change at various time intervals was calculated by $2^{-\Delta\Delta CT}$. EF1α was selected as internal control for normalization.

Statistical analysis

The differences in transcript levels between different time intervals were tested for statistical significance using one-way ANOVA followed by Duncan's new multiple range test using the statistical package, (SAS Inc., USA) (Attwood *et al.*, 2007; Alenjandro *et al.*, 2008) p value below 0.05 was considered statistically significant. The results were expressed as the mean±SD.

Results and Discussion

Semi quantitative analysis of gD gene in time course study

The expression pattern of gD gene in BHV-1 infected cell line was investigated in a time course study. The expression pattern varied between different time intervals. Expression was observed lower in RT-PCR analysis in 0 to 3 h.p.i while increased expression was observed in between 6hr to 12hr expression. The expression showed an static trend from 12 to 24 h.p.i. (Figure 1). The expression pattern was compared with that of EF1 - α , whose expression remained unchanged throughout the time period (Figure 2).

Real time expression profiles of glycoprotein D gene in MDBK cells of BHV-1 infected

Relative expression analysis of the glycoprotein D gene at different time points post BHV-1 infection was also studied by Real Time RT-PCR using SYBR green chemistry. B actin was used as the internal control. In Real time analysis it was observed that there was a tremendous increase in the expression of gD gene in between 6hr to 12 h.p.i. expression followed by a static expression at 24 h.p.i (Figure 3). A fold change of greater than 45000 fold was observed in the expression gD at 12 h.p.i. and then showed a gradual stability. The expression of EF1 - α remained same throughout the experimental period. Melt curve analysis of the genes showed a single peak for all the genes studied and expression levels were found to be statistically significant.

The BHV-1 genome encloses ten genes encoding the glycoproteins, among them, six genes are in the UL region, namely gK (UL 53), gC (UL 44), gB (UL 27), gH (UL 22), gM

(UL 10), gL (UL 1) and four remaining genes are in the US region, gG (US 4), gD (US 6), gI (US 7) and gE (US 8). BHV-1 infection of permissive cells is initiated first by the low affinity interaction between viral gB and/or gC to the cell surface structures like heparin sulfate sugar moieties (Li *et al.*, 1995). This is followed by the stable binding of BHV-1 gD to cellular specific receptor and subsequent virus penetration by fusion of the virion envelop with the plasma membrane (Liang *et al.*, 1989). Glycoprotein D (gD), a major viral immunogen, is essential for virus replication and is responsible for inducing the strongest immune response, reducing virus replication and shedding by host. The gD gene is well studied and highly conserved among herpes viruses. It is located in the US region between map units 0.892 and 0.902 of the BHV-1 genome, encoding a 71 kilodalton (KDa) glycoprotein of 417 amino acids (aa), containing both N- and O-linked oligosaccharides (Chase and Letchworth, 1994, Tikoo *et al.*, 1993). These properties of gD make it an excellent candidate for genetic characterization of the Egyptian vaccinal strain (Abu-hammad) of BHV-1.

As no reports were found on the functional aspects of gD, the present study was designed to check whether the gD gene expression profiles in BHV challenged *Mdbk cells*. The expression levels of glycoprotein D gene was quantified by normalizing with the expression of β actin. The gD gene expression was observed at all the time points tested including the control i.e., the unchallenged group. The expression was almost constant till 3 h.p.i. while a significant increase was observed in between 6 to 12 h post infection (h.p.i). The expression static from 12 to 24h.p.i. A similar study by Hassan Momtazl and Mohammadreza Nazem, (2010) in in bacterial cell revealed that the expression of infectious bovine rhinotracheitis virus. According to them Bovine Herpesvirus 1 (BHV-1) belongs

to the genus of Varicellovirus and the family of Herpesviridae which contains three main gB, gC and gD genes. In order to cloning of the coding region of gD gene of IBR virus, PCR product of the open reading frame of the gene from IBR virus isolated in Iran was amplified by PCR. A 1047bp PCR product of the gD gene with EcoRI, HindIII restriction sites were subcloned of pTZ57R/T and digested by the mentioned endonucleases. Digested insert cloned in topET-32a and transferred in *E. coli* cells. For the expression of gD protein, the pET-32a recombinant vector was transformed and then induced in BL21 (DE3) strain of *E. coli* competent cells using IPTG. The presence of gD expressed protein was shown in immunoblotting and SDS-PAGE system. With respect to the remarkable frequency of infection to IBR in Iran and the necessity of controlling it through vaccination with recombinant vaccines of thymidine kinase, manufacturing and applying the recombinant gD protein are vital goals in recognition and distinction between infection and responses caused by vaccine.

The detection of viral infections may be based on direct or indirect diagnostic method. The development of molecular biology has contributed to the appearance of highly sensitive new diagnostic approaches. Various recombinant DNA techniques have been applied to the rapid detection of nucleic acid, used to study viral genes and also to produce new types of vaccines. PCR has been one of the most rapidly adopted techniques. Reports on the application of PCR to the diagnosis of infectious diseases are also accumulating at an exponential rate (Erlich *et al.*, 1991; Pershing *et al.*, 1991). Molecular virology has served to establish bovine herpes virus (BHV-1) as the prototype member of ruminant herpes viruses based on the genomic sequence of the virus.

The nucleotide sequence of the BHV-1 genome (136 kb) has completed by

international cooperation (July 1995; it comprises 67 unique genes and 2 genes, both duplicated, in the inverted repeats. BHV-1 genome encodes approximately 73 proteins but only 54 transcripts have so far been identified in productive infection by northern blot analysis (Wirth *et al.*, 1989). There are at least 10 glycoprotein with predicted molecular weights range from 17 -101 kDa. They are generally N – linked and O – linked glycosylation with homodimer and heterodimer forms. These virally encoded glycoproteins are gB (UL27), gC(UL44), gD (US8), gI(US7), gH(UL22), gL(UL1), gG(US4), gK(UL53), gM(UL10). gB (UL27) contain 932 amino acids with MV 130 KDa essential for attachment, entry, cell to cell spread and fusion (Byrne *et al.*, 1995). gC contain 508 amino acids with MW 91 KDa responsible for variable attachment (chase *et al.*, 1993), gD(US6) with 417 amino acids with MW 71 KDa essential for entry and cell to cell spread, gE (US7), 575 amino acids (MW 92 KDa) cell to cell spread and virulency. UL11, UL36, UL37, UL41, UL46, UL47, UL48, UL49 and US9 are the major tegumental proteins (Schwyzer *et al.*, 1996). Capsid proteins are UL18, UL19, UL26, UL35 and UL38. DNA replication proteins are UL 29 (major DNA binding protein), UL30, ul42 (DNA polymerase), UL5, UL8, UL29 (helicase) (Geng *et al.*, 1995).

Glycoprotein D(gD), a major viral immunogen, is essential for virus replication and is responsible for inducing the strongest immune response, reducing virus replication and shedding by host (34). The gD gene is well studied and highly conserved among herpesviruses. It is located in the US region between map units 0.892 and 0.902 of the BHV-1 genome, encoding a 71 kilodalton (KDa) glycoprotein of 417 amino acids (aa), containing both N- and O-linked oligosaccharides (Abdelmagid1 *et al.*, 1998) (Fig. 4 and 5).

Fig.1 gD gene expression at different time points post infection. Lane M: 100bp plus marker, Lane 1-4: h post infection 0, 3, 6, 12, 24 h.p.i, respectively

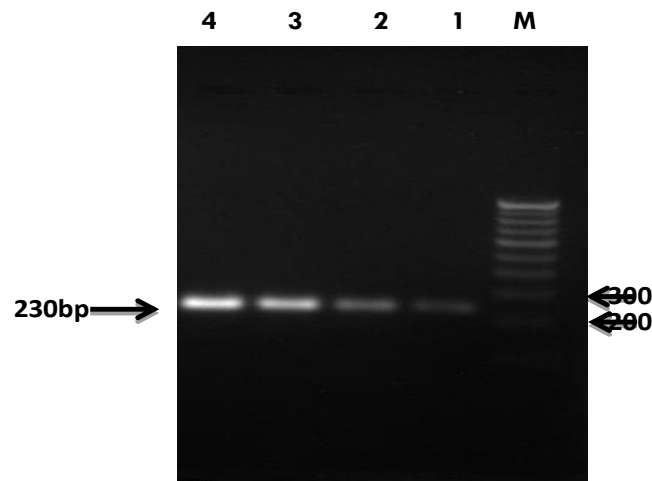


Fig.2 β actin expression at different time points post infection. Lane M: 100bp plus marker, Lane 1-4: h post infection 0, 3, 6, 12, 24 h.p.i, respectively

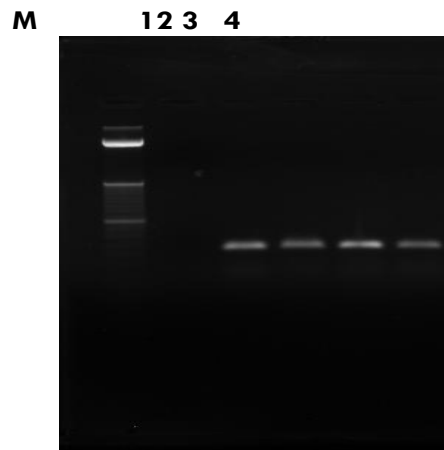


Fig.3 Amplification curve showing Expression profile of gD gene in MDBK cells after infection at different hours

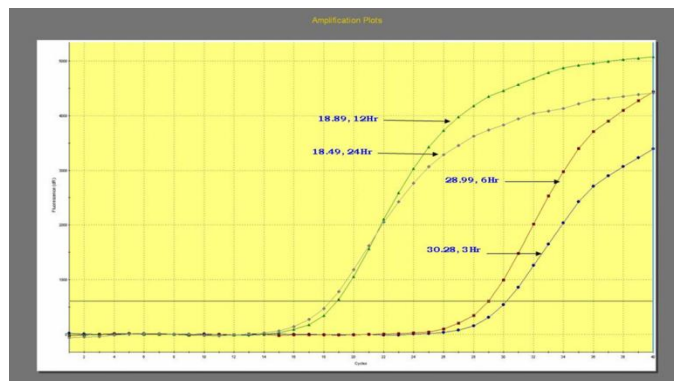


Fig.4 Dissociation curve showing specific amplification with a single peak

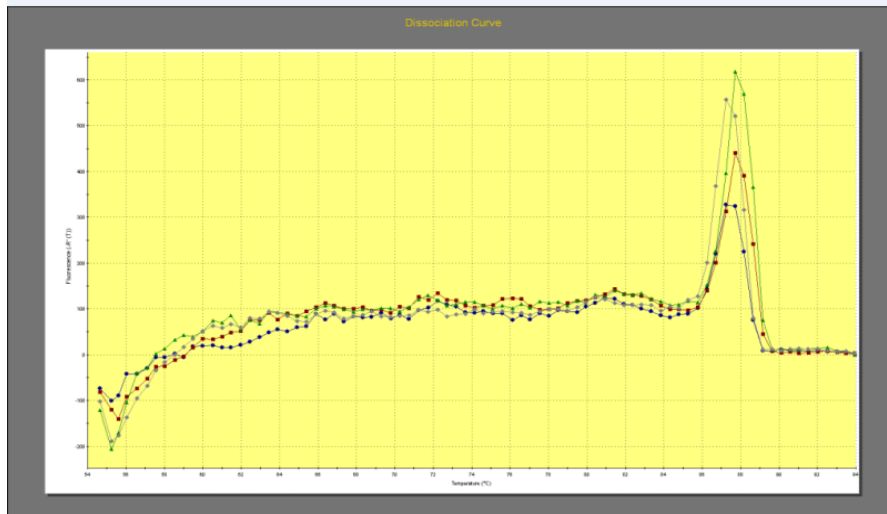
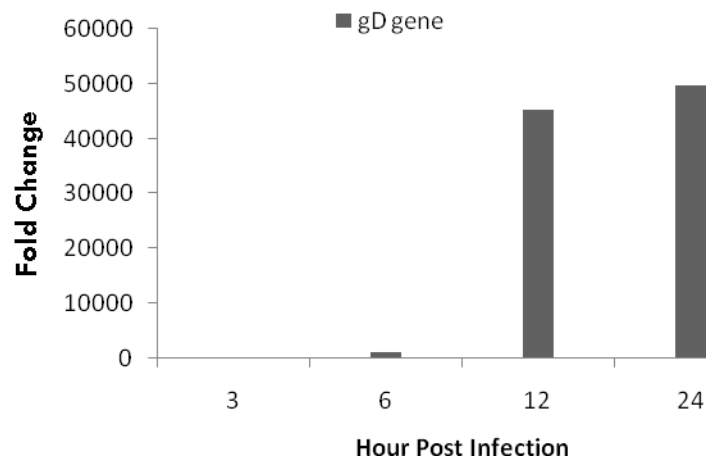


Fig.5 Fold changes (mean±SD) in expression of gD gene in real time PCR



These properties of gD make it an excellent candidate for genetic characterization of the Egyptian vaccine strain (Abu-hammad) of BHV-1. because of their location in the virion envelope and on the surface of infected cells, the glycoproteins are important targets for the host immuneresponse. Furthermore, they play important roles in pathogenicity mediating entry of the virion into the host cellfusion, and cell -to-cell spread of virus Glycoprotein D (gD) of bovine herpesvirus 1 (BHV-1), a homolog of herpes simplex virus gD, represents a major component of the viral envelope and is a dominant immunogen.

To study the antigenic properties of the different regions of gD, O.Y. Abdelmagid have expressed the full-length gD encoding gene and overlapping fragments spanning various regions of the gD open reading frame in abaculovirus (*Autographa californica* nuclear polyhedrosis virus) – insect cell (*Spodoptera frugiperda*, SF-9) system. Maximum levels of expression for all proteins were obtained 48 to 72 h post infection of SF-9 cells by recombinant viruses. Full-length and truncated recombinant gD proteins reacted specifically with anti-gD monospecific serum as determined by immune precipitation

and immunoblotting, indicating that the proteins retained their antigenicity. However, based on the reactivity with a panel of gD-specific monoclonal antibodies (Mabs), the full-length recombinant gD lacked proper expression for two highly neutralizing linear epitopes identified by Mabs R54 and 9D6. The rest of the epitopes appeared to be preserved and antigenically unaltered. Immunofluorescence studies of recombinant baculovirus infected SF-9 cells using gD monospecific serum. Revealed no direct correlation between cellular localization of the expressed proteins and their amino acid sequences (Abdelmagid *et al.*, 1998).

At different time interval the total RNA was isolated from BHV -1 infected MDBK cell lines treated with DNase to free genomic DNA contamination of RNA which was used as template in PCR with BHV-1 specific primers there was no amplification in PCR which conformed the RNA was free of BHV-1 genomic DNA. This RNA used to pfor DNA synthesis by oligo (dT) primer using MMLV reverse transcriptase at 45 C. this cDNA was used for real time PCR with gD specific BHV-1 primers at 58 C temperature. The objective was to understand the expression profile of gD gene of BHV-1 virus in MDBK cell line after infection.

The present study reveals novel report on the expression profile of gD gene at transcript level in BHV-1 infected MDBK cells. gD gene predicted to encode a glycoprotein envelope protein and its expression patterns observed in the current study reveals the importance of this gene in BHV-1 pathogenesis by virtue of their role as virus envelope protein.

However, further studies need to be undertaken to unfold the molecular mechanism of these virus host interactions to be able to design an effective therapy against this dreaded disease of shrimp.

Conflict of Interest

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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How to cite this article:

Asmita Singh and Ajay Kumar. 2018. Expression Profiling of Glycoprotein D Gene of Bovine Herpes Virus in Madindar by Bovine Kidney Cells via Real Time PCR. *Int.J.Curr.Microbiol.App.Sci.* 7(10): 2807-2816. doi: <https://doi.org/10.20546/ijcmas.2018.710.326>