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Expression of Immunodominant gI Protein of Duck Enteritis Virus and its Evaluation for ELISA

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ABSTRACT

Background: Duck plague (DP), also known as duck viral enteritis (DVE) caused by *Anatid alpha herpesvirus-1*, is the major infectious viral disease reported in India very often with significant economic losses due to high morbidity and mortality. The genome is approximately 158,091 bp with a genomic arrangement pattern (UL-IRS-US-TRS) that corresponds to type-D herpesvirus. The incubation period of the disease in domestic ducks ranges from 3 to 7 days and the mortality varies from 5-100%. It has been reported that the strategies to monitor and control DVE were often frustrating because the disease tends to establish a latent infection in waterfowl and vaccination failure. The present study was taken as an attempt to develop indirect ELISA using developed recombinant gI protein to diagnose DEV in field condition.

Methods: In this study, we selected a membrane glycoprotein protein I (US7) gene, that plays an important role in virion sorting, cell-cell spread and binding of IgG Fc receptor. The gI protein was cloned with pET-32a (+) and expressed in a prokaryotic (*E. coli*) expression system. Further, recombinant protein was purified by nickel column affinity chromatography and refolded by dialysis. The obtained concentrated protein was then evaluated for its antigenicity and reliability in an Indirect-Enzyme-linked immunosorbent assay (ELISA) for DEV antibody detection in duck sera.

Result: A panel of positive, negative and vaccinal serum was evaluated which indicated the potential use of the protein in the development of a serological assay for sero- surveillance of duck plague infection.

Key words: Duck enteritis virus, Duck viral enteritis, ELISA, Glycoprotein I, Prokaryotic expression, Recombinant protein.

INTRODUCTION

Across the globe, duck raising, for meat and eggs, has become a lucrative practice, next to chicken rearing. Being low maintenance, it is quite popular in many Asian countries including India (Rajput *et al.*, 2014). Duck Viral Enteritis is caused by *Anatid herpesvirus 1* or Duck Enteritis Virus (DEV). It is classified under the genus *Mardivirus*, subfamily *Alphaherpesvirinae* and family *Herpesviridae* of order *Herpesvirales* as per the report of virus taxonomy, 2011 by ICTV (International Committee on Taxonomy of Virus) (King *et al*., 2011). Duck plague is another term used commonly in place of DVE, coined by Bos in 1942 and was first used officially by Jansen and Kunst in 1949. Other synonyms are Entenpest in Germany, peste du canard in France, eendenpest in Denmark and duck plague in the USA (Davison *et al*., 1993). It is a lethal, acute, or chronic, contagious disease of geese, swans and ducks (Davison *et al.,* 1993, Dhama *et al*., 2017) and is not found to harm members of other mammalian and avian species. The disease is reported globally in domestic and wild waterfowl and has more economic impact in duck-rearing countries such as Europe, Africa, Asia and North America. The disease is known to spread across the continent by migratory birds. *Anatidae* family (geese, swans, ducks) are the natural hosts for this virus (Liu *et al.,* 2018; Chang *et al.,* 2018; Sarmah *et al.,* 2020). Natural outbreaks with huge casualties have occurred in different age groups, in a range of 7 days to adult age of the various breeds of domestic ducks (*Anas platyrhynchos*) including Indian Runner, White Pekin, Khaki Campbell and mixed breeding ducks, Muscovy (*Cairina moschata*) (Wozniakowski and Samorek-Salamonowicz 2014; Dhama *et al*., 2017).

The first outbreak of DEV was reported by Baudet in 1923 in Netherland and confirmed by DeZeeuw in 1930 (Friend and Pearson, 1973; Dhama *et al.,* 2017). In 1967, another outbreak was reported in Long Island, New York in the duck industry which led to the loss of $1 million (Plummer *et al.,*1998). Later it was reported in the Fingerlake region

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of New York in various waterfowl species thus making it an enzootic disease in the USA (Plummer *et al.,* 1998). The disease has also been reported in different countries including China (Jansen and Kunst, 1964), India (Mukherji *et al.,* 1965), England (Asplin, 1970), Vietnam and Germany (Kaleta, 2007). The disease occurred only in Muscovy ducks in the USA (Davison *et al.,* 1993; Campagnolo *et al.,* 2001).

DEV genome contains a globular, bilayer-lipid envelope with large genome of 120-130 nm and 158 kb (Chen *et al.*, 2021) and icosahedral symmetry (Gardener *et al.,* 1993). DVE infection occurs by direct or indirect interaction with an infected bird or environment (Sandhu and Shawky, 2003). Natural virus transmission occurs by water-borne channels. Infected tissues can be administered via parenteral, intranasal, or oral routes to create an experimental infection. In wild birds, a carrier condition is suspected. Recovered birds become latently infected carriers. The DVE virus, like other herpesviruses, may fall into latency and the trigeminal ganglion appears to be a latent location for the virus. Survivors from the natural outbreak exhibited viral shedding up to 4 years of infection. Many, resistant to disease also serve as a carrier of the virus (Plummer *et al*., 1998).

Presently there is no therapy available for this disease. Vaccinations are the only available remedy for this disease. Preventive measures need to be taken to prevent entry of virus in the flock.

Currently, clinical signs and lesions are used as a presumptive diagnostic means and several laboratory methods are used for confirmatory and differential diagnoses. Various laboratory techniques available are virus isolation, virus neutralization, molecular methods including PCR, in-situ hybridization using a specific oligonucleotide probe (Cheng *et al*., 2008) and LAMP-based nucleic acid amplification (Ji *et al.,* 2009; Jiang *et al*., 2012). Neutralizing antibodies against DEV is suitable to use as a tool in the diagnostic assay (Aravind *et al*., 2012; Dhama *et al*., 2017). The immunological tests commonly used are FAT, virus neutralisation assay and reverse passive hemagglutination test (RPHA) (Sandhu and Shawky, 2003). Out of these, FAT is considered the second most sensitive, next to virus isolation. Indirect ELISA for DEV is preferred to detect the antibodies against the duck plague virus (Neher *et al.,* 2019). Recombinant protein-based ELISA can prove to be efficient in diagnosis of DEV. Hence, the present study was aimed at construction of recombinant gI protein and to use it in development of ELISA.

MATERIALS AND METHODS

Virus

Virulent Duck Enteritis Virus isolate (DEV/INDIA/IVRI-2016), preserved in a lyophilised form in the Immunomodulation laboratory, Immunology section, ICAR-IVRI have been utilized in this study. DNA was isolated from tissue samples using the phenol-chloroform method.

Construction of recombinant plasmid

N-glycosylation sites were predicted using the NetNGlyc-1.0 online tool (https://services.healthtech.dtu.dk/service.php?NetNGlyc-1.0). The whole genome sequence of the Indian isolate (DEV/India/IVRI-2016) (NCBI Accession MZ824102.1) is available which was utilised for design. The amino acid sequence of gI were subjected to N-glycosylation site prediction. All primers were designed using the NCBI primer blast tool (https:// www.ncbi.nlm.nih.gov/tools/primer-blast/) for amplification of gene regions containing the immune dominant region of the virus. Full-length US7 gene was amplified from the isolated DNA template using the designed oligo primers (gIF-ATGCGGATCC ATATATGATGATTGCCC and gIR-ATGCAAGCTT CTGCATATCATCAGAT). The amplicons of PCR were subjected to restriction enzyme digestion using enzymes BamHI and HindIII (at above bold site of the sequence). These digested amplicons were then ligated to prokaryotic expression vector pET32a which has been digested with same enzymes (BamHI and HindIII) using T4 DNA ligation kit (Takara, Japan). E. coli JM109 competent cells were transformed with the above-mentioned ligated mixture after an overnight ligation at 4°C and the transformants were then examined on ampicillin- containing LB agar plates. Colonies were found in pET-32a (+) following an overnight incubation at 37°C. Colony PCR was done to confirm the presence of transformed cells using the same restriction enzymes BamHI and HindIII. The presence of bands of specific size confirmed the transformation. The PCR-positive colonies were diluted into LB broth and cultured overnight at 37°C and 180 rpm in a shaker incubator. Plasmids were taken out of the overnight cultures and RE digested with BamHI and HindIII to examine them.

Retransformation of the recombinant plasmid into the expression host

E. coli BL21(DE3) pLysS was used as the new expression host for the purified plasmids. As chloramphenicol is the resistance marker for the expression host and ampicillin is the resistance marker for the vector, the transformed cells were next plated on the LB ampicillin-chloramphenicol agar plates and incubated overnight at 37°C.

Expression and detection of target proteins

Initial expression was generated on a limited scale for the positive recombinant colonies for each area identified by colony PCR and RE digestion. Three colonies were chosen from the total colonies that surfaced after overnight incubation and cultured in LB broth with ampicillin and chloramphenicol at 37°C for 12 hours. The overnight grown culture was then reinoculated in LB broth and incubated at 37°C with 180 rpm shaking for around 3 hours until the OD of the bacterial growth reached 0.6. and the bacterial growth was triggered by adding IPTG at a final concentration of 1 mM.

The cultures were collected six hours after induction and allowed to continue growing at 30°C. Following STB

treatment, the pelleted cells were analysed by SDS-PAGE examination to determine the amount of expression and particular molecular weight of the expressed proteins.

Western blotting was used to further examine these expressed proteins and assess their reactivity. The Penta His-HRP conjugate was easily able to identify the transferred protein bands on the membrane and it was able to distinguish them from the negative sera since no discernible bands could be observed in blots that had been exposed to negative duck serum.

The expressed proteins were then purified using denaturing conditions. Ni-NTA columns were used in metal affinity chromatography to separate the expressed proteins. The level of purity of the isolated protein was then evaluated once again using SDS-PAGE. After being dialyzed against PBS to eliminate any remaining traces of reducing agents, imidazole, or salts, these purified proteins were then concentrated with PEG 20000. Then, to keep the concentrated, purified proteins from degrading, trehalose and protease inhibitors were added.

Standardization of ELISA

96 well ELISA plate was coated with 1:500 dilution of purified concentrated gI protein of DEV in Carbonate-Bicarbonate buffer (coating buffer) and incubated at 4°C overnight. Ten-fold serial dilution (1:10 to 1:80) of known infected, negative and challenged duck sera were prepared in the blocking buffer (3% LAH in 0.05% PBST) to finalize the serum dilutions. ELISA plate is washed 3 times with wash buffer (0.05% Tween-20 in PBS) without any hold time. Each well of the ELISA plate was added with 50 µl of diluted sera sample in duplicates. It was then incubated at 37°C for 1 hour. The plate was then washed using PBST wash buffer 3 times followed by discard. 3 washes were done with a hold time of 3 minutes in each wash. The polyclonal anti-duck HRP-conjugate antibodies were diluted to 1:3000 in the blocking buffer and 50 µl was added to each well. The plate was then incubated at 37°C for 1 hour. Plates were washed using wash buffer 3 times having 3 minutes of hold time at each wash. Substrate solution was added 50µl in each well. The plate is incubated at 37°C for 15 minutes. The reaction was stopped using 50µl of stop solution in each well. Reading was taken at 492 nm in an ELISA reader. To validate the developed ELISA assay, diagnostic sensitivity and diagnostic specificity were analysed. The known status of birds before sampling was used for the categorization of the samples.

RESULTS AND DISCUSSION

The gene sequence determined for the Indian DEV isolate (DEV/India/IVRI-2016) was retrieved from the NCBI (GenBank Accession number MZ824102.1). It was used as template to amplify US7 gene (gI protein) starting from 270 to 750 aa (513 bp). After digestion with BamHI and HindIII the DNA fragment was inserted into the vector digested with same enzymes to yield recombinant plasmid for expression. The product was expressed in an *E. coli* system, *E. coli* BL21 (DE3) pLysS. Initial expression was generated on a limited scale for the positive recombinant colonies for each area identified by colony PCR and RE digestion. The bacterial growth was triggered by adding IPTG at 1Mm concentration. Following STB treatment, the cells were analysed using SDS-PAGE and Western blot technique. The presence of 37 kDa protein band confirmed the presence of the expressed recombinant gI protein. The proteins were then purified under denaturing conditions using Ni-NTA columns in metal chromatography to separate expressed proteins. Purified proteins were then concentrated with PEG 20000 and trehalose and protease inhibitors were added to prevent purified proteins from degrading.

The best antigen concentration to be utilised was determined by the checkerboard titration using the gI proteins produced in the pET-32a (+) vector and purified under both native and denaturing conditions. The successive two-fold dilution of the known positive serum (28 dpi) and the known negative serum (ranging from 1:10 to 1:80) also helped determine the best test serum dilution. Based on I-ELISA, it was concluded that a uniform test serum dilution of 1:20 was appropriate because it offers the greatest range of absorbance values for known positive and negative sera at the preset concentration of gI protein. Protein concentration of ~20 ng/ well (1:500 dilutions of the stock solution) optimum OD values of positive controls between 1-1.5. Therefore, this concentration was finalised. OD value was decided in range for positive and negative samples. For positive samples, the range of OD value was decided to be 0.9-1.54 while for negative samples, the OD range was between 0.2-0.46. The cut-off value was decided as 0.5 (3X to the background control). The detection of positive samples and negative samples was 100%. The rate of detection of vaccinated birds was 75%.

Glycoprotein (gI) protein is the structural protein located in the unique short region of the viral genome. The gene US7 codes for the gI protein, which further encodes for the membrane protein. gI protein has an important role in promoting cell-to-cell spread. It is a type I membrane protein complexed with gE. ORF of gI gene is 1116 bp in length and 371 amino acids polypeptide is its primary translation product. gI gene is expressed when the virion is enveloped indicating that it is a late viral gene that takes part in envelope assembly to form mature DEV virions (Li *et al.,* 2011). The current study was undertaken to clone and express the gI protein of DEV in a prokaryotic expression system.

Viral DNA was subjected to PCR with desired gene sequence. Primers designed were such that they had the recognition sequences for BamHI and HindIII to facilitate the directional cloning of the US7 region of the gene and expression of gI protein. After RE digestion, for directional cloning the vector pET-32a (+) was used. The vector was digested with the same pair of enzymes that was used as insert; PCR amplified products. The RE digested products were then subjected to overnight ligation at 4°C. The ligation mixture was then transformed into JM109 competent cells of *E. coli* strain. Colonies appearing after overnight

transformation procedure was further screened for the presence of transformed cells by colony PCR and RE digestion methods. Recombinant protein after confirmation was purified and retransformed into *E. coli* BL21(DE3) pLysS. Then, the colonies that had undergone transformation were plated onto an LB agar plate containing ampicillin and chloramphenicol. All the prokaryotic expression vectors used in this investigation include the ampicillin resistance gene, whereas the expression host cells BL21(DE3) pLysS carry the chloramphenicol resistance gene, hence these two antibiotics were chosen as the markers. The recombinant gI protein was expressed as His-tagged fusion protein. SDS-PAGE was used to examine these recombinant proteins to determine their unique molecular weight and degree of expression. When amino acid residues from the vector were added to the actual size of the protein, the observed size of the His-tagged fusion proteins on SDS-PAGE was essentially identical to the estimated size. Western blotting was used to examine the expressed proteins in greater detail. Under the denaturing conditions, Ni-NTA metal affinity chromatography was used to purify the expressed proteins. These pure proteins were concentrated with PEG2000 after being dialyzed in PBS to eliminate any excess reducing agents, imidazole, or salts.

For standardization of ELISA, based on the checker-board titration, the ideal antigen concentration and serum dilution were determined. Based on the findings of the checkerboard titration, the final concentration of the antigens and serum was set at 20 ng/well and 1:20 dilution for I-ELISAs based on recombinant gI. 100% positive samples reacted with the protein whereas 100% negative samples did not produce any OD. No reactivity could be observed with unvaccinated and un-infected samples.

CONCLUSION

As the DEV is a herpes virus, there is probability of latent infection in the birds. Hence, a companion diagnostic assay is essential to implement the control programme with vaccination. The currently developed indirect ELISA showed promising results to discriminate the infected and un-infected birds. The assay will be handy for establishment of sero-surveillance before vaccination programme can be implemented. The recombinant protein expressed in this study using prokaryotic system showed the assay is highly inexpensive, helping the economically implementation of the programme. No indigenous kit is available for the diagnosis of DEV hence this will be first kit of such kind developed in the country. The same protein will further be utilised for development of antigen detection immunodiagnostic assays.

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Conflict of interest

The authors declare no conflict of interest.

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