

## Isolation and Rapid Identification of a Kerala Isolate of Duck Enteritis Virus using Polymerase Chain Reaction

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(Received: 08 July 2013; accepted: 19 October 2013)

Duck enteritis virus (DEV) is a herpes virus that cause an acute, contagious, and fatal disease. In this present article, we introduce a polymerase chain reaction (PCR) assay for DEV DNA. The method employed primer sets targeting the viral DNA polymerase gene (UL30), type I membrane protein that binds to heparin sulphate (glycoprotein C) and glycoprotein E of DEV and was able to amplify DNA fragments of the expected size from infected samples. The method will provide a valuable tool for a rapid laboratory diagnosis of DEV infection. By virtue of its high throughput format, the method may be useful for large epidemiological surveys and clarification of pathogenesis, such as latency of the virus.

**Key words:** Duckenteritis virus, DNA polymerase, Glycoprotein C, Glycoprotein E Polymerase Chain Reaction.

Duck viral enteritis (DVE) also known as duck plague, is a fatal viral infection of ducks, geese, swans and other species of the order Anseriformes<sup>1</sup>. The etiological agent, duck enteritis virus (DEV) or Anatid herpes virus-1 (AHV-1) is a member of *Alphaherpesvirinae* family<sup>2,3</sup>. DVE was first diagnosed in the western hemisphere in a commercial duck producing area of Suffolk County, New York<sup>4</sup> and since then DVE epizootics are reported in USA<sup>5,6</sup>. The disease has spread in many countries throughout the world causing heavy mortality in domestic ducks and wild mallards. It has been frequently recorded in the Netherlands where it first appeared in 1923. The disease has also been reported from Belgium<sup>7</sup>, Britain<sup>8</sup> Vietnam and other South East Asian countries<sup>8</sup>.

DUCK plague (DP), or duck virus enteritis is one of the major infectious diseases of ducks causing considerable mortality in India since 1963. In Kerala state, several DP outbreaks have been confirmed despite regular vaccinations<sup>9</sup>. Thereafter, the disease has spread in different parts of the country and frequent outbreaks have been recorded in the duck-rearing areas including Kerala<sup>10</sup>, Tamil Nadu<sup>11</sup>, West Bengal<sup>12</sup> and Assam<sup>13</sup>. DVE causes significant economic loss in duck production in many places of India and the socio-economic group most affected by the disease is the small and marginal farmer.

The genome of DEV VAC strain is 162,175 bp in length with 44.91% G+C content<sup>14</sup>. Nucleotide sequence analysis indicate that the genome is composed of two unique sequences, unique long (UL) and unique short (US), the latter being flanked by inverted repeat elements (IRS and TRS) of 13,029 bp each<sup>14,3</sup>. The UL region of the virus, extending from position 1 nt to 119,305 nt, contains 65 probable protein coding genes. Starting from the left end of the genome, the arrangement of the genes is mostly collinear with HSV-1 and MDV.

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Fifty-eight genes in the UL region have homologs in genus *Alphaherpesvirinae*<sup>14,3</sup>. The US region of the DEV genome, extending from position 132,336 nt to 145,062 nt, is predicted to encode 9 genes. Of them, 7 are homologs of the genes US10, US2, US3, US4, US6, US7, US8 of HSV-1, respectively, and one is a homolog of MDV gene SORF3. Only DEV US5 has no homolog in other Herpesviruses<sup>3</sup>. Flanking the US region, the RS regions consist of IRS and TRS, both of which are 13,029 bp in length and contain 2 genes, ICP4 (infected cell protein 4) and ICP22 (US1)<sup>3</sup>.

Duck plague is difficult to monitor and control because the virus establishes an asymptomatic carrier state in water fowl that is detectable only during periods of intermittent virus shedding<sup>15</sup>. Although avian tissue culture systems are routinely used to isolate duck plague virus, they are not always reliable indicators of virus presence because cell cultures vary in sensitivity and are less sensitive than animal hosts<sup>16,17</sup> whereas PCR has advantage of rapidity, sensitivity and robustness for diagnosis.

## MATERIALS AND METHODS

### Cells and virus

Chicken embryo fibroblasts (CEF) were cultured at 37°C with 5% CO<sub>2</sub> in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin. DEV virulent strain was isolated from the clinical samples collected from Kerala. For this study we collected liver, spleen and intestine samples of dead birds from different parts of Kerala. Triturated samples were inoculated in both 25 and 75 cm<sup>2</sup> flasks in 3.0 ml quantities and allowed to adsorb for 1 hr at 37°C in 5% CO<sub>2</sub> atmosphere. Five and ten millilitres of growth medium containing M-199- supplemented with 10% foetal calf serum, 2mM L-glutamine, and antibiotics (100 U/ml penicillin, and 100 µg/ml streptomycin) was added to each flask, and the flasks were incubated until infected cells reached 90-100% cytopathic effect (CPE). Flasks were then frozen and thawed three times, and the cell debris was pelleted by centrifugation at 800g for 20 min. The supernatant representing the virus stock was subdivided into vials and stored at -80°C.

### Genome preparation

CEFs infected with sample fluid were mechanically dislodged from the flask surface. Cells and extracellular particles were pelleted by centrifugation at 16,000X g for 30 min. Pelleted cells were disrupted with sterile distilled water by freeze thawing three times, then centrifuged at 16,000X g for 5 min, and the pellet was discarded. The supernatant was centrifuged at 16,000X g for 30 min to concentrate virus particles. Pellets were resuspended in 200 µl Tris-EDTA buffer (pH 8.0), and 10 µg of ribonuclease A was added before incubation at 37°C for 30 min. Proteinase K was added to final concentration of 0.2 µg/ml, and the mixture was incubated at 56°C for 1 hr. The preparation was made 1% for SDS and incubated at 37°C for 30 min, and then NaCl was added to 0.5 M. The mixture was extracted two times with ether. Samples were made 2.5M for ammonium acetate, and the DNA was precipitated with two volumes of 95% ethanol held at 21°C for 30 min. The preparation was centrifuged at 16,000X g for 30 min, and the pellet was washed once with 70% ethanol. Viral DNA pellets were resuspended in 40 µl of TE buffer and stored at 4°C. We confirmed the purity of the DNA sample by taking the 260/280 OD ratio, it was 1.8. Thus our DNA isolate free from RNA and protein contamination.

### Designing of primers

We have designed three sets of primer for the identification of the duck enteritis virus from the DNA isolated from the cell culture. For the designing of primers we used the sequence data that is available in NCBI (EU082088.2). The designing of primer was done manually and we analyzed the primer sequence using available bioinformatics software. Three sets of primers were designed to amplify the conserved regions of the proposed DEV UL30 (DNA polymerase), UL 44 (gC), and US 8 (gE). The primer set 1 for UL30 forward primer (BamH I) 5' CCGGATC CATGGCAGAGTCCGGGTAGAAAC3' and UL30 Reverse primer (Sal I) 5' CCGTCGA CTCACC GTTTATCTTAACCTTAC3'.

The proposed size 1596 bp.

The set 2 primer pair was designed for UL 44 (gC) Forward primer (BamHI) 5' CCGGATCCATGGG CCATTAGTGATGGTTGCCTTC3'

Reverse Primer (XhoI) : 5' CCCTCGAGTC AAATATTGTCTGCTTTATCTC3'

and the proposed size was 1296 bp.

The primer set three were designed for US 8 (gE)  
Forward primer (BamHI) 5' CCGGATCC  
ATGCGCGCGTGTTCGATTAT 3'

Reverse primer (Hind III) 5'  
CCAAGCTTTTACACTTCCCCGGGCGACG 3'

and the proposed size was 1473 bp.

#### Polymerase Chain Reaction (PCR)

All three sets of primer were tested for their ability to produce amplicons. Primer sets were prepared according to the manufacturer's recommendation and optimized to 100 ng/ µl of DNA. Genomes of DEV field isolates were diluted to 200ng/µl of DNA material. The DEV field isolate DNA was tested with the three sets of primers. The PCR products from the first and second primer sets were sequenced and the BLAST analysis and MEGALIGN through DNA STAR programme.

Samples were subjected to the following in an automated DNA thermal cycler. The initial cycle was 94 C for 5 min, Step 2: 94°C for 1 min, Step 3: 55°C for 1min, Step 4: 72°C for 1.5 min, (Steps 2 to 4 were repeated for 30 cycles), Step 5: 72°C for 10 min

For the confirmation of the PCR product, after PCR amplification, electrophoresis was carried out with 10 µl of PCR product in 1.2% agarose gel along with a 100 bp ladder plus marker.

## RESULTS

#### Isolation of DNA and PCR

The suspected field samples for DEV were used for virus propagation in the primary culture of CEF cells. Infected primary culture was observed for 3-4 days for occurrence of CPE. Initial passaging of the virus showed very less prominent CPE. Subsequent passaging of the virus produced more prominent CPE within 48 hrs of infection and at faster rate compared to initial passages.

Cytopathic changes produced, pyknotic rounding that resulted in very small grape like clusters of affected cells within 24 to 36 hours (Fig.1&2). The foci then enlarged slightly and some became necrotic. The cells infected with DEV virus were harvested for viral DNA isolation.

Culture flask with more than 80% CPE was frozen at -20°C and freeze thawed three times to harvest the supernatant. The stock virus was kept in aliquots. One of the aliquot was titrated in CEF culture and the titre was calculated following the method of Reed and Muench (1938). And from the above data we calculated the titration of the virus in the sample is  $10^{6.5}$  TCID<sub>50</sub>/ml.

The DNA extracted from CEF culture had an OD 260/280 ratio equal to 1.8, indicating DNA to be pure which was used for the amplification of the selected three genes. The result of PCR reaction showed bright and intense band of amplicon. Agarose gel electrophoresis of PCR product produced expected band length for three genes. The band length observed was UL30-1596 bp (Fig.1), gC -1296 bp ( Fig.2) and gE-1473bp (Fig.3).

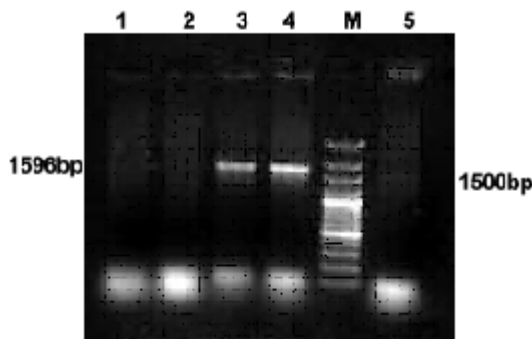
## DISCUSSION

The disease was first reported from Kerala in 1980. To our knowledge this report provides the first sequence information for DEV from Kerala<sup>9</sup>. In Kerala there are three indigenous breeds of duck populations. For this experiment we collected samples of the three breeds from different parts of Kerala. To the date there is no assays have been developed for the specific detection of the Kerala isolate.

The PCR assay developed for the duck viral enteritis offers a rapid, specific, and sensitive method for detecting and identifying DEV virus in field isolate from without detecting other avian

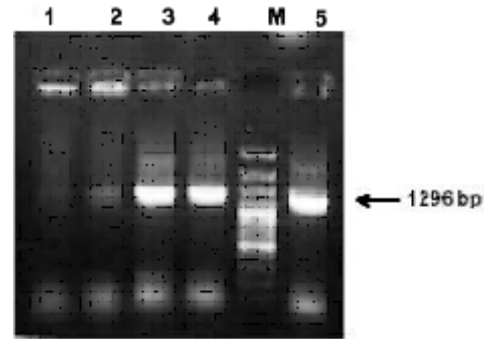
**Table 1.** Three sets of primers

No	Primer	Sequence (5'-3')
1	Forward	CCGGATCCATGGCAGAGTCGGGTAGAAAC
	Reverse	CCGTCGACTCACCGTTTATCTTAACCTTAC
2	Forward	CCGGATCCATGGGGCCATTAGTGATGGTTGCCTTC
	Reverse	CCCTCGAGTCAAATATTGTCTGCTTTATCTC
3	Forward	CCGGATCCATGCGCGCGTGTTCGATTAT
	Reverse	CCAAGCTTTTACACTTCCCCGGGCGACG



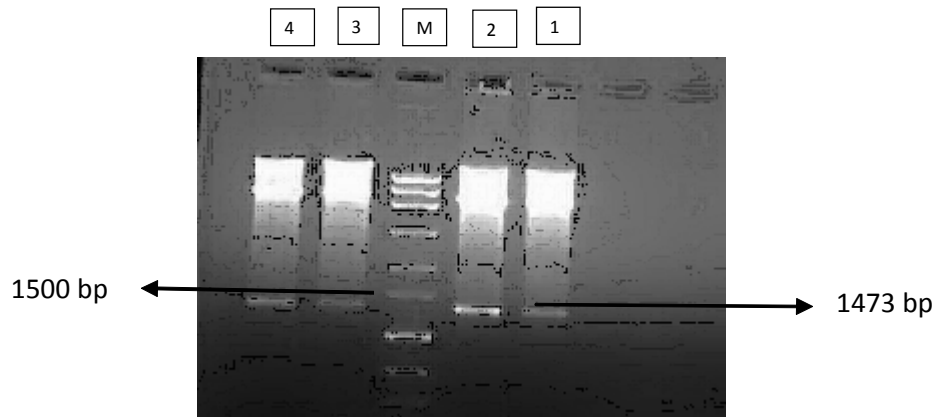
Lane M :100bp DNA marker  
Lanes 3& 4:UL30 amplicons showing 1596 bp  
Lanes 1, 2 &5 : Negative clones

**Fig. 1.** The amplification of UL30 gene of DEV using the primer set 1



Lane M : 100bp DNA marker  
Lanes 1,2,3 & 4 : UL 44 (gC) amplicons showing 1296 bp  
Lanes 5: Negative clones

**Fig. 2.** The amplification of UL44 (gC) gene of DEV using the primer set 2



Lane M:1Kbp DNA ladder  
Lanes 1, 2, 3& 4 : US8 (gE) amplicons showing 1473bp

**Fig. 3.** Amplification of gE gene of DEV virus using primer set 3

herpes viruses<sup>18</sup>. On the basis of the comparisons made, the oligonucleotide primers selected for this PCR were specific for DEV virus. In the present study we designed primers for three specific genes in the DEV virus, the DNA polymerase, gC and gE of the above virus. The UL region codes for the DNA polymerase gene (UL30). The UL region especially UL 30 is highly conserved in alpha herpes iverinae family<sup>3</sup>. It shows only 55%, 58% and 53% identity with HSV-1, MDV, and PRV respectively<sup>3</sup>.

The gC belongs to the unique longue regions (UL-44) of the virus, and it shows only 24%, 30% and 28% identity with HSV-1, MDV, and PRV respectively<sup>3</sup>. The third candidate belongs to the unique short (US-8) regions of the DEV virus, and it codes glycoprotein E (gE). It shows only 27%, 23% and 26% identity with HSV-1, MDV, and

PRV respectively<sup>3</sup>. These results suggest that the DEV primer sets may not be good for detecting other herpes viruses.

The PCR found 20 times more sensitive than tissue culture for detecting DEV virus when the number of infectious units per sample was compared with the number of gene copies<sup>18</sup>. These PCR characteristics, and a quick sample processing than traditional virus isolation and serologic identification methods used for detecting DEV virus.

#### ACKNOWLEDGMENT

The authors are thankful to the Director, Indian Veterinary Research Institute for providing the facilities to carry out the work

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