STANDARD OPERATING PROCEDURE			
Title:	**Duck Plague Virus(DPV)- Detection of DPV DNA by gel based PCR**		
Reference:	**OIE Terrestrial Manual, (2012) with modification by S. Neher**	*Author:*	**Dr N N Barman, Dr S. Neher**

1.0 INTRODUCTION

1.1 Purpose/Scope of this SOP

This SOP outlines the method for the detection of DPV DNA in blood and tissue samples. DNA is isolated from samples using the QIAGEN QIAamp® Viral DNA mini-kit or the Nucleo-Puro DNA Sure Tissue Mini Kit(Qiagen and Genetix); following which DPV specific DNA sequences are detected using a commercial gel basedPCR method.

1.2 Background Information

Gel based PCR method is one of the most sensitive technique for DNA detection currently available. By using specific OIE primers as detailed in OIE protocol, a one-step PCR method has been developed.

2.0 MATERIALS

CONSUMABLES	**SUPPLIER**
0.2ml PCR tubes	GENAXY
EQUIPMENTS	**SUPPLIER/ MODEL**
Class 2 safety microbiological safety cabinet	LABCHEM & LABORTENIK INSTRUMENTS
Micro-centrifuge	TARSONS
VERITI 96-well Thermalcycler	APPLIED BIOSYSTEMS
Micropipette (0.5-10 µl, 10-100 µl, 20-200 µl, 100-1000 µl)	GENAXY (Nichipette)
Filter Tips	GENAXY
QIAamp® Viral DNA mini-kit	QIAGEN

3.0 PROCEDURES

3.1 Collection of Duck Plague suspected samples

Clinical as well as post mortem samples were collected from field outbreaks of DP for detection of the duck plague virus (DPV).

3.1.1 Clinical samples:

Clinical samples like Cloacal swabs and whole blood were collected from suspected cases of duck plague. Swabs were collected in sterile tube (Himedia, India) containing sterile Hank's balanced salt solution and whole blood was collected in EDTA. The collected samples were transported in ice and stored at -20^0C until further use.

3.1.2 Post mortem samples:

Ducks that died of suspected DPV infection were subjected for post mortem examination and all organs were thoroughly examined for presence of typical DP lesions. Samples like liver, Spleen, kidney, oesophagus were collected in sterile vials without adding any preservatives as well as in 50 percent phosphate buffer glycerine and transported to the laboratory in ice.

3.2 Detection of Duckplague viral nucleic acid:

Clinical as well as post mortem samples collected from affected ducks were processed for detection of viral nucleic acid by polymerase chain reaction. Standardization of Polymerase chain reaction was done following the method described by OIE *Terresterial Manual*, (2012). In DPV genome, DNA directed DNA polymerase gene was targeted and anticipated product size was 446bp.

3.2.1 Standard duck plague virus:

A vaccine strain of DPV was procured from A.H &Vety. Biological, Govt

of Kerala and used as known positive control to compare with unknown

field samples.

3.3 Extraction of viral DNA:

For extraction of DP viral DNA various samples were prepared following standard methods:

- Freeze dried duck plague virus vaccine was reconstituted in sterile distilled water.
- Field tissue samples (liver, spleen, kidney, oesophagus) were homogenized and 20 percent suspension was prepared by suspending in appropriate volume of PBS (pH7.4)
- Cloacal swabs squeezed in HBSS and whole blood collected from DP suspected birds were used as such for extraction of viral DNA using Tissue and blood DNA extraction kit (Qiagen and Genetix).

3.3.1 A standard protocol enclosed in the kit was followed:

- Briefly, a volume of 70µl of tissue homogenate, 180µl buffer ATL and 20µl proteinase-K was taken in 1.5ml micro centrifuge tube.
- Mixture was vortexed for 2 minutes and incubation was done at 56^0C for 10 minutes.Repeatation of the vortex and incubation was done again.
- Vortex of the tube was done and added buffer ATL 200µl. Again vortex was done and ethanol 200µl (96 to 100 percent) to the micro centrifuge tube and vortexed again.
- Transferring of the material was done from micro centrifuge tube to spin column and then spinned was done at 8000 rpm for 1 minute.
- Collection tube was discarded and transferred the spin column to another collection tube and addition of the buffer the AW_1 500µl was done and again spinned at 8000 rpm for 1 minute.
- The collection tube was discarded and spin column was transferred into another collection tube and addition of the buffer AW_2 500µl was done.
- The collection tube containing the reagents was transferred and spinning was done at 1000 rpm for 5 minutes and the collection tube was discarded.
- The spin column was placed in 1.5 ml micro centrifuge tube and 200µl buffer AE

was put into the membrane of spin column.Incubation was done at room temperature for 1 minute.

- Finally, centrifugation of the micro centrifuge was done at 8000 rpm for 1 minute and then sediment was collected.
- Extracted DNA samples were properly labelled and stored at -20^{0}C until use.

3.4 Polymerase Chain Reaction (PCR):

The PCR was standardized using freeze dried duck plague vaccine as known DP viral DNA to amplify DNA directed DNA polymerase gene. Amplification of the targeted gene was done using the following reaction condition and primer sets (Table.1).

TABLE1. PRIMERS USED TO AMPLIFY DNA-DIRECTED DNA POLYMERASE GENE.

Primer	Sequence (5' – 3')	Position
Primer-1 Sequences (Forward)	**5'-GAA-GGC-GGG-TAT-GTA-ATG-TA-3'**	55520
Primer-2 Sequences (Reverse)	**5'-CAA-GGC-TCT-ATT-CGG-TAA-TG-3'**	55965

3.4.1 Reaction master mixture:

The targeted gene of the DP virus was amplified using following reagents (Table2).

TABLE 2: DETAILS OF THE PCR REACTION MIXTURE

Total reaction volume : 50µl	
Reagents/Ingredients	Volume
Nuclease free water	33.5µl
10X buffer	5µl
25mmol MgCl2	3µl
10mmol dNTP	1µl
Forward primer (20pmol)	1µl
Reverse primer (20pmol)	1µl
Taq polymerase 5unit/1µl	0.5µl
DNA template	5µl

The contents were mixed by brief spinning and placed all the tubes in a thermal cycler (Applied Biosystems, USA) using following thermal cycling conditions (Table 3):

TABLE3 : DETAILS OF THE THERMAL CYCLER CONDITION

Number of cycles	**Temperature**	**Time**
One cycle	94^0C	2 minutes
	37^0C	1 minute
	72^0C	3 minutes
35 cycles	94^0C	1 minute
	55^0C	1 minute
	72^0C	2 minutes
One cycle	72^0C	7 minutes
	4^0C	Stored until use

3.5 <u>Confirmation of PCR amplicons</u>:

The amplified PCR products was confirmed by agarose gel electrophoresis using 1.7 percentagarose (Amresco) containing ethidium bromide in 0.5X tris borate EDTA(TBE) buffer.

- Briefly, gel casting tray was set up by placing the comb in the slot containing 8 wells.
- A volume of 40ml agarose gel was prepaerd by adding 0.680g of agarose to 40ml of 0.5X TBE.
- The agarose was melted in microwave oven (Haier, India) for 1 minute.
- Thereafter, the molten agarose was allowed to come down the temperature for about 56^0C and 1 µl of ethidium bromide (10mg/ml) was added.
- After proper mixing it was poured on the gel casting tray and allowed to solidify without disturbing the tray.
- Then 0.5X TBE was poured on the gel casting tray till the gel was submerged and the comb was removed carefully.
- Then 10 µl of each PCR amplified products was mixed with 2 µl of 6X loading dye (Fermentas) and loaded into the wells.
- Along the PCR amplicon a 100bp DNA ladder (Fermentas) was loaded and run parallel to the PCR amplicons.
- Electrophoresis was carried out at 78V for 1hour and 30 minutes. The gel was then visualized on a UV transilluminaor (Kodak, Biostep, Germany).

3.6 Interpretation

Samples positive for duck plague virus showed 446bp sized band whereas in negative sample there was no such band.