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MOLECULAR AND IMMUNOLOGICAL DETECTION OF KENCHU VIRUS INFECTING SILKWORM (*Bombyxmori* L)

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ABSTRACT

In India, silk textile is economically important industry and Karnataka alone accounts for 63% of the country's silk production. The silk industry is affected by many fungal and viral major diseases and thus there is decrease in the silk production. In this study the early detection of the viral disease caused by Kenchu virus to silkworm (BmKV) was studied by molecular and immunological methods. The Kenchu virus isolate was collected from Vijayapura, Karnataka, India and ultrapurified using sucrose gradient centrifugation. The DNA was isolated from ultrpurified virus using standard protocol. The PCR detection of BmKV was done using DNV1 and DNV2 gene specific primers where, the isolate of Kenchu virus collected from Vijayapura, Karnataka, India amplified ~700 bp band specific to DNV2 primer and belongs to Japanese or Yamanashi isolate and DNV1 primer did not amplify. The antibodies were raised against the purified virus and immunologically Dot-ELISA and plate-ELISA were standardized to check the specificity of antibodies raised against the BmKV.The titer 1:50 of antigen, 1:16000 of primary antibody and 1:1000 of secondary antibody dilutions was found optimum for detection of the virus with BCIP/NBT as substrate for Dot-ELISA and for plate ELISA 1:100 dilution of crude and ultrapurified antigen and 1:1000 dilution of both primary antibody (antisera raised against BmKV) and secondary antibody was optimum with $OD_{405\ nm}$readings of1.041 and 1.03 respectively. This study thus helps in development of immunodiagnostic kit for Kenchu disease detection and help farmers to avid heavy losses of silkworm production.

KEYWORDS: Bombyxmori, Kenchu Virus, PCR, Dot-ELISA, Plate ELISA

INTRODUCTION

India is the world's second largest producer of textile and garments. India earns about 27% of its total foreign exchange through textile exports. Further, the textile industry of India also contributes nearly 14% of the total industry production of the country. Indian textile industry can be divided into several segment are cotton, silk, woollen, jute and coir textiles. Silk textile is one of economically important industry. Silk is favored over other types of fibres due to its extraordinary properties like water absorbency, heat resistance and efficiency of dyeing [Deori et al., 2014]. Silk is called as "queen of fabric" because of its luster, softness, color, biodegradability, elegance, biocompatible, strength and flexible properties. Silk was first discovered in China between 2600 - 2700 BC and it produces 80.06% of total silk in the world. India is considered as second largest producer of silk after China i.e. about 17.77% total worldwide silk production. Eri, Mulberry, Muga and Tasar are four commercially available silk in India [Mazzi et al., 2014].

India's Production of mulberry silk is about 17,746 tonne per year, while production of non-mulberry silk is about 120 tonne per year. Silk is spun by larvae of the silkworm. Art of culturing silkworms or sericulture is an important agro based industry in India particularly Karnataka which accounts for 63% of the country's silk production followed by 22 % in Andhra Pradesh and 5 % in Tamilnadu. About 90% of total silk production in India is carried out in these three states [Kuneez, 2013]. Sericulture is an important foreign exchange earning agricultural occupation; it is highly labour intensive and hence has the potential of providing labour to the poor and rural masses. In sericulture industry successful production of cocoon depends on the disease management because silkworms are infected by many pathogens like bacteria, fungus, protozoan and virus [Nirupama 2014].

Most common diseases cause economical loss to sericulture industries [Kaufmann et al, 2011] are Infectious Flacherie (Sappe), Densonucleosis (Kenchu), Nuclear polyhedrosis (Grasserie or NPV), Muscardine, Pebrine, etc. Among these, viral diseases are difficult to control and needs management by early detection of infection. Densonucleosis (*Bombyxmori*Kenchu virus(BmKV) or *Bombyxmori*Densoculeosisvirus (BmDNV)) disease is most frequently occured and causes more loss with death of silkworms, affecting the silk production.

Densonucleosis virus belongs to the subfamily called Densovirinae and Parvoviridae family. The symptoms of *Bombyxmori* L. Kenchu virus infection look apparently normal just after infection but slightly dull and redness of skin will be observed after 2 hours. Sometimes thoracic region looks swollen. In later stage of infection, vomiting gut juice and feces with high moisture content is noticed (Fig. 1). The DNV virus is a linear single stranded DNA virus (according to the Baltimore classification DNV are II group viruses). These viruses infect only insects and not plants and they also acts on only invertebrate. Examples of viruses which come under these genus are *Aedesalbopictus*densovirus,*Junoniacoenia*densovirus and *Galleria mellonella*densovirus. DNVs are grouped into two genera Iteravirus (BmDNV-1 and BmDNV-5) and Bidensovirus (BmDNV-2, BmDNV-3 and BmDNV-4) which have bipartite genome) with two sets of complementory DNAs namely viral DNA-1 (VD1) and viral DNA-2 (VD2) [Meng et al., 2010].The management of the virus is very much important and methods like chemical treatment and other resistance breeding methods are expensive, hazardous and complicated. So, an alternative for these approaches in the use of biotechnology driven tools like developing immunodiagnostic kit and developing customized strategies based on the molecular characterization of casual organism.

This study was thus undertaken with the objectives of molecular and immunological detection of the Kenchu virus infecting silkworm (*Bombyxmori* L.).

MATERIALS AND METHODS

Research pertaining to the Molecular and immunological detection of Kenchu virus infecting silkworm (*Bombyxmori* L.) was carried out in the Department of Biotechnology, University of Agricultural Sciences, GKVK, Bengaluru

Purification of the Kenchu Virus (KV)

KV infected silkworms samples were collected from silkworms rearing field in Vijayapura (Karnataka, India) based on symptoms of infection by Kenchu virus. Purification of Kenchu virus (KV) was done as per as the procedure of Patil*et al*. (1992), with some modification (Figure 2).

Extraction of Viral DNA

The procedure given by Sambrook et al. (1989) was employed with some modification. The precipitate of virus obtained by sucrose density gradient centrifugation was dissolved in 250 µl of TE buffer (10 mMTris-Hcl: 1.0 Mm EDTA) pH 8.0, in an autoclaved Eppendorf tube. To this 10µl of 0.5 M EDTA , 1.5 µl of Protinase K (to a final concentration of 50 µg /ml) and 12.5 µl of 10% SDS (Sodium dodecyl sulfate), were added, mixed well by vortexing and kept in a heating block maintained at 56°C for 1 h. The mixture was then cooled to room temperature, to which 260 µl of phenol – chloroform (1:1) was added and mixed by inverting several times. The mixture was spun in a microfuge at 15,000 rpm for 10 min. at room temperature.The upper phase was removed carefully and the Phenol–Chloroform extraction was repeated thrice to get a clear interphase. Finally, to the lower phase equal volume chloroform: Isoamylalcohol (24:1) was added and mixed well and centrifuged in a microfuge at 12,000 rpm for 15 min. at room temperature. To the upper phase of this (300 µl), about 30 µl of 3 M sodium acetate, pH 7.0 and 600 µl (double the volume) of ethanol were added, Mixed gently and kept at -70°C for 1 h. for precipitation of DNA. Following this, the mixture was centrifuged at 15,000 rpm for 15 min. at 4°C to obtain the pellet of DNA. The pellet was washed twice with 70% ethanol, dried in vacuum to remove the traces of ethanol. The dried pellet of DNA was dissolved in a few microlitres of TE buffer, pH 8.0 and stored at -20°C. The isolated genomic DNA was quantified and purity was determined by measurement of absorbance at 260 and 280 nm using biospectrometer (Eppendorf). The DNA (5 µl) isolated from different cardamom genotypes was electrophoresed on 0.8% agarose gels, stained with 0.5 µg/ml ethidium bromide, visualized and photographed under UV light in a gel documentation system (Alpha Innotech Corp., USA).

PCR Detection of Densonucleosis Virus

The primers specific to Densonucleosis virus (DNV1 and DNV2) were synthesized based on chemical characteristics and sizes of structural proteins, further amplified with suitable annealing temperatures. The reaction was carried out in 25 µl reaction volume containing 20 ng genomic DNA, 1 U Taq DNA polymerase (Bangalore GeNei), 2mM dNTPs, 10X PCR reaction buffer with 1.5 mM MgCl2 and 5 picomole of each forward and reverse primers as per the standardized protocol (Awasthi et al., 2008). Amplification conditions consisted of initial denaturation at 94°C for 2 min, denaturation at 94°C for 1 min, annealing at 53 °C (DNV1) and 54 °C (DNV2) for 2 min, extension at 72°C for 3 min and final extension at 72°C for 7 min with 40 cycles. The amplified products were electrophoresed on 1 % TAE agarose gel and captured in Alpha innotech gel documentation unit.

Table 1

Primer	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
DNV1	AGAGGTGAACACGAAGAATA	GGCGTGAAGTATGTGGAAAT
DNV2	GAAGATACTGTCCCAAATGA	CCTTTCAGGTTTGCTTCTTCTTG

Immunological detection of BmKV

Production of Antibody against BmKV Virus

Polyclonal antibodies were raised against BmKV virus in New Zealand white rabbits [van Regenmortel, 1982].

Immunization Schedule

The rabbit was immunized following routine procedure. Antibody production was primed using Freund's complete adjuvant and subsequent injection and the booster were administered in Freund's incomplete adjuvant as shown in following flow chart (Fig.3).

Indirect Dot-ELISA for the Detection of BmKV

The basic principle involved in immunochemical techniques is that a specific antigen will combine with its specific antibody to give an antigen-antibody complex. This complex is usually insoluble and cannot be seen with the naked eye. The reaction is made visible with the help of a colour reaction, where the secondary antibody is conjugated with an enzyme and made to react with a substrate (Clark and Adams., 1977).

Dot-ELISA technique was carried out to detect the virus and to confirm that the antibody raised against BmKV was specific to virus (Smith and Banttari., 1987). The concentrations of antigens used were 1:10, 1:20 and 1:50 dilutions. The dilutions of primary antibody used were 1:500, 1:1000, 1:2000, 1:4000, 1:8000, and 1:16000 with secondary antibody dilutions of 1:1000, BCIP (5-bromo-4-chloro-3-indolyl-phosphate) in conjunction with NBT (nitro blue tetrazolium) as substrate for colour development.

Indirect Plate ELISA for the Detection of BmKV

The antiserum titer was determined using secondary antibody alkaline phosphatase conjugate (Clark and Adams., 1977).The 1:10, 1:20, 1:50 and 1:100 dilutions of crude and ultrapurified antigen were used with healthy control and secondary antibody dilution of 1:1000. The antiserum dilutions used were 1:500, 1:1000, and 1:2000 with pNPP (para-Nitrophenylphosphate) as substrate for colour development.

RESULTS AND DISCUSSIONS

The present study was carried out in the Department of Biotechnology, University of Agricultural Sciences, Bangalore, India. The objectives of the study were presented in the following subheadings:

Purification of Kenchu Virus

The Kenchu virus diseased silkworms were collected from field in Vijayapura, Karnataka, India and purified by sucrose density gradient. Quantification of the ultrapurifiedvirus sample from infected silkworm sample was measured using biospectrometer (Eppendorf). The concentration of the virus was 256 µg/ml, which was sufficient to trigger the immune system of rabbit to produce polyclonal antibody against the BmKV virus. The DNV isolates from Nistari, C'nichi, NB1 and Guangnnong Marked (GNM) were purified using Cesium chloride gradient centrifugation, following the standard methods (Awasthi et al., 2008).

DNA Isolation from BmKV

DNA isolation was done with the ultrapurifiedKenchu virus and the quantification was done using biospectrometer (Eppendorf) measured at $A_{260\ nm}$/ $A_{280\ nm.}$ The DNA concentration of 86 µg/ml was obtained and used for PCR detection using DNV 1 and DNV 2 gene specific primers of BmKV.The purity of DNA was checked by running on the 0.8 % agarose gel electrophoresis (Fig 4). Similar study was done by Awasthi et al. (2008)where, DNA was isolated

from the Kenchu virus infected haemolymphusing QiagenDNeasy Tissue kit.

PCR Detection of Densonucleosis Virus

The DNV1 and DNV2 BmKV gene specific primers were amplified with 20 ng/µl DNA and only DNV2 primer was amplifiedwith ~700 bp band size for the isolate collected from Vijayapura, Bangalore, Karnataka, India. DNV1 primer did not show any amplification (Fig 5). The DNV2 primer amplified for Vijayapura isolate of Kenchu virus may belong to Japanese or Yamanashi isolate of DNV infection and DNV1 primer not amplified may be due to its resistance to DNV1 infection (Awasthi et al., 2008).

Serological Detection of BmKV

The antibodies were raised against the isolated and purified Kenchu virus by giving the intramuscular injections with booster doses. The rabbit was bled after final booster and the serum containing antibodies was collected and stored at -20°C.

Dot-ELISA was standardized with all the combinations as mentioned above in the material and methods, there was colour development except the buffer and healthy control. The titer 1:50 of antigen, 1:16000 of primary antibody and 1:1000 of secondary antibody dilutions was found optimum for detection of the virus with BCIP/NBT as substrate (Fig. 6).

In plate ELISA colour development was observed in all the combinations except in control. Absorbance was read at OD_{405nm} in the ELISA reader (Table 1) and the optimum reading of 1.041 was observed in the combination of 1:100 dilution of crude antigen and 1:1000 dilution of both primary antibody (antisera raised against BmKV) and secondary antibody, also optimum OD reading of 1.03 was observed in the combination of 1:100 dilution of ultrapurified antigen and 1:1000 dilution of both primary (antisera raised against BmKV) and secondary antibody.

CONCLUSIONS

In the present experiment the Kenchu virus was ultrpurified using sucrose gradient centrifugation and resulted in true characteristic symptoms of the disease. DNA was isolated and standardized from the ultrapurifiedKenchu virus using standard protocol. The molecular detection of the BmKV using gene specific primers DNV1 and DNV2 was carried out and DNV2 primer alone was amplified with ~700 bp specific to Japanese isolate of DNV infection. The ultra-purified virus and crude virus were used as an antigen for serological diagnosis such as polyclonal antibody production against BmKV, Plate -ELISA and DOT-ELISA were standardized and successfully used for detect the virususing goat anti-rabbit IgG alkaline phosphate as conjugate. The present study thus helps in development of an immunodiagnostic kit for the detection of Kenchu virus and thus help the farmers to avoid heavy losses of silkworm production.

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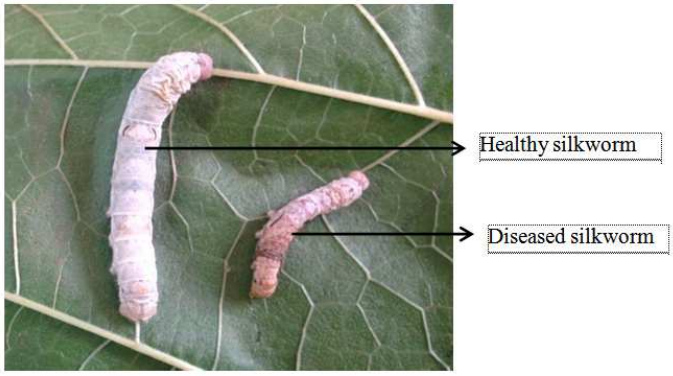


Figure 1: Healthy and Kenchu Virus Infected Silkworms

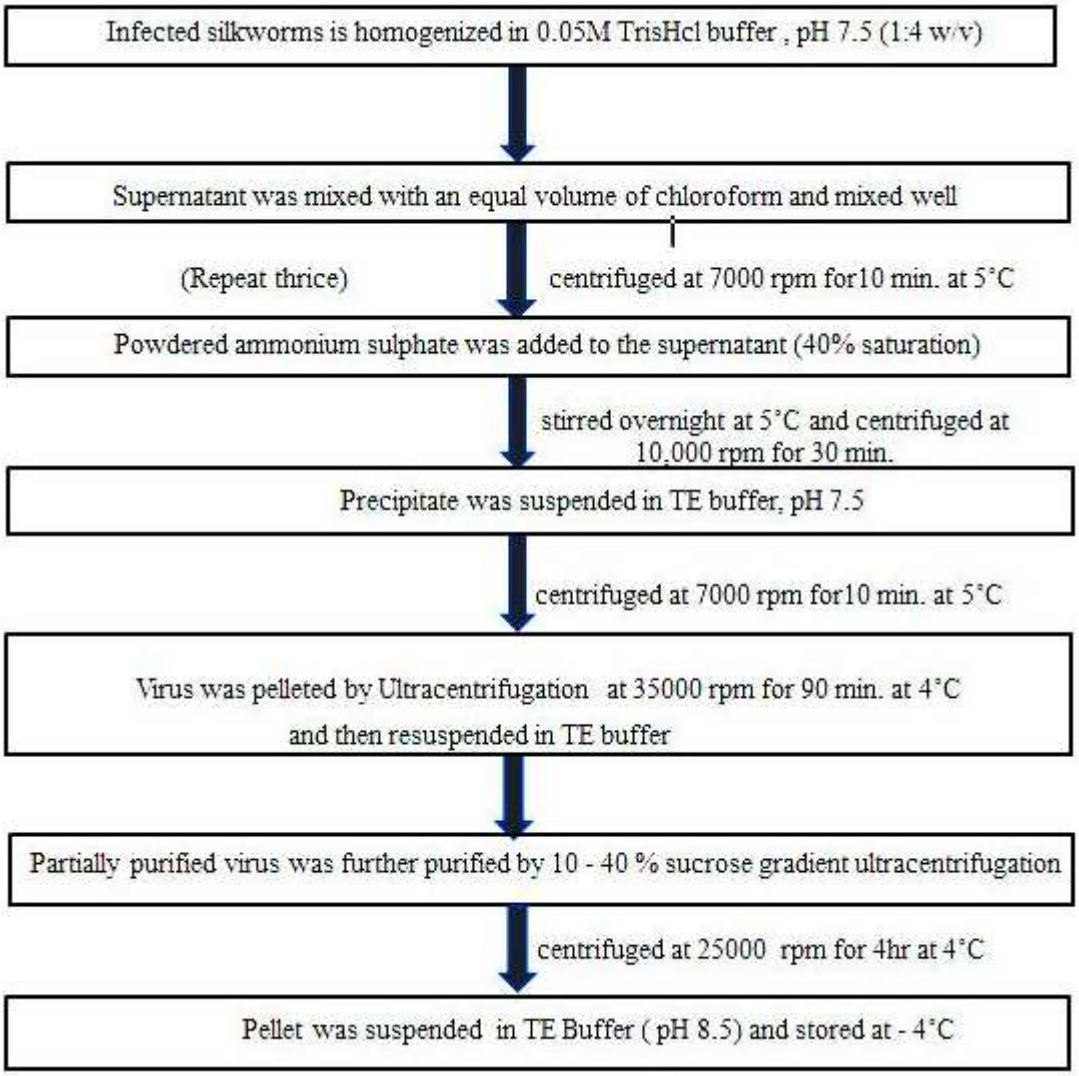


Figure 2: Protocol for Purification of *Bombyxmori*kenchu Virus (Bmkv)

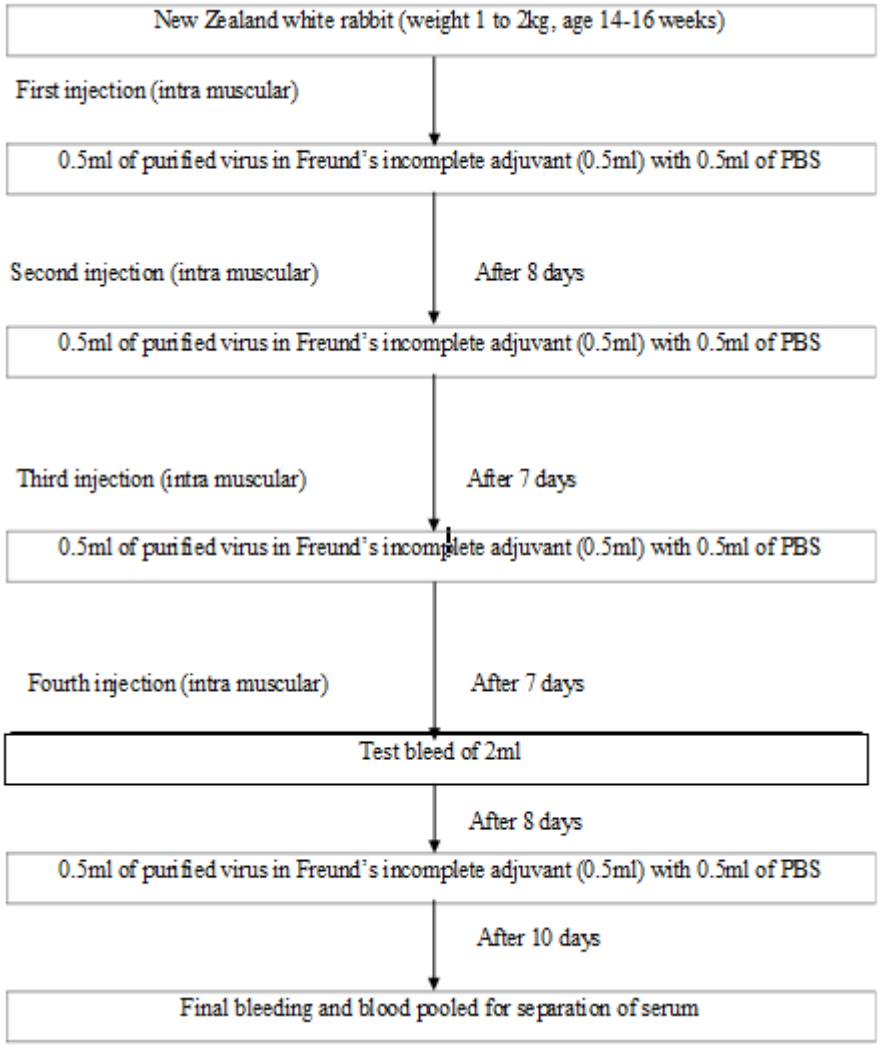


Figure 3: Immunization Schedule for Rising of Antibodies Against Ultrapurifiedkenchu Virus

Figure 4: Gel Picture Showing the DNA Isolated From the Ultrapurified Virus

Lane 1: DNA isolated from the ultrapurifiedKenchu virus

Lane 2: DNA ladder 250 bp

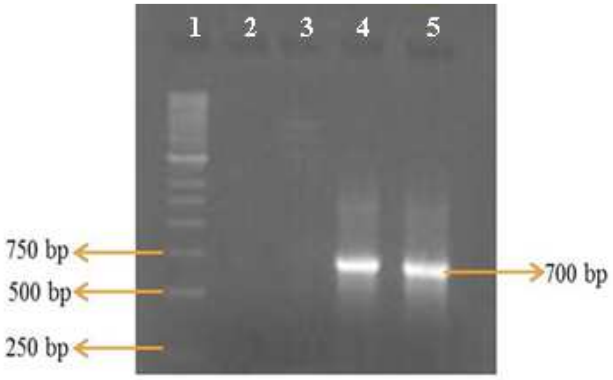
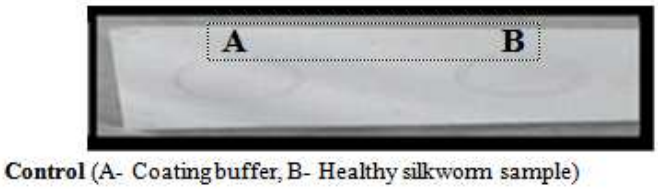


Figure 5: PCR Detection of Bmkv Using Gene Specific Primers

Lane 1: DNA ladder 250 bp

Lane 2 and 3: DNV 1 gene specific primer amplification

Lane 4 and 5: DNV 2 gene specific primer amplification



Control (A- Coating buffer, B- Healthy silkworm sample)

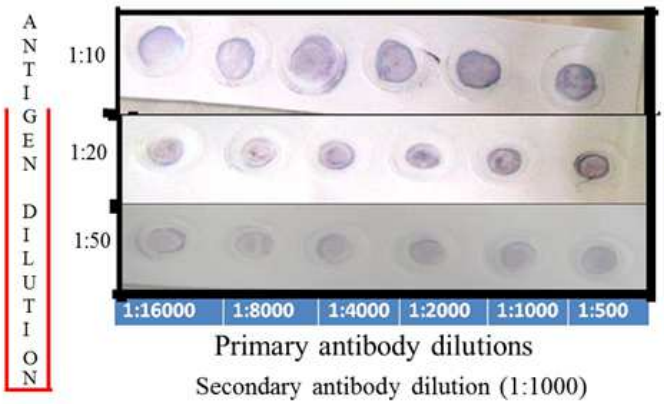


Figure 6: Standardization of Antibody Titer Raised Against BmKV by DOT-ELISA Forultrapurified Virus

Table 1: Plate ELISA Readings at $OD_{405\ Nm}$ for the Detection of Bmkv against the Raised Antibodies

		Crude Antigen Dilution				Ultra Purified Antigen Dilution			
Primary Antibody Dilutions		**1:10**	**1:20**	**1:100**	**Control**	**1:10**	**1:20**	**1:100**	**Control**
	1:500	1.123	1.141	1.186	0.031	1.147	1.632	1.608	0.018
	1:1000	1.153	1.134	**1.041**	0.040	1.899	1.341	**1.030**	0.024
	1:2000	0.303	0.411	0.521	0.034	1.231	1.132	0.812	0.021
Secondary Antibody Dilution 1:1000									