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Comparative Polypeptide Profiling: Isolation, Propagation and Purification of Indian Isolates of Buffalopox Virus

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Abstract: Buffalopox is a contagious viral disease-affecting buffaloes (*Bubalus bubalis*) and rarely cows, with morbidity up to 80%in affected herd causing high economic losses to the farmers. In the present study, as a preliminary work, the field isolates of BPV recovered from suspected clinical materials obtained from outbreaks of different geographical locations of the country were propagated in Vero cells and purified along with reference BP4 virus for comparison. Purification of BPV was carried out in sucrose density gradient (60-36%), where two white opalescent bands appeared after ultracentifugation. The upper band, above the 36% sucrose layer was found to be enveloped virus (Extra cellular enveloped virus) where as the band at interface was the pure naked virus (Intracellular Mature Virus). The purity of the virus preparations was assessed by UV-spectrophotometry, SDS-PAGE and infectivity assay in cell culture. The extinction ratio values (O.D. 260/280) for all the BPV isolates were within the range of 1.2 to 1.4 indicating highly purified nature of the BPV. On the basis of polypeptide compositions, BPV isolates were similar and contained more than 25 polypeptides with a molecular weight range from 14.2 kDa to 180 kDa. Both the opalescent bands produced characteristics CPE in Vero cell line in infectivity assay. This preliminary study will help in undertaking further work on protein profile and further characterization, which, in turn, will help in understanding the virological and immunological properties of the new virus isolates for development of suitable immuno-diagnostics and prophylactics.

Key words: Buffalopox virus, Indian isolates, isolation, propagation, purification

INTRODUCTION

Buffalopox is a contagious viral disease-affecting buffaloes (*Bubalus bubalis*) and rarely cows, with morbidity up to 80% in affected buffaloes herd. Disease results in high economic losses due to reduction in the productivity (milk and meat) and working capacity of affected animals. The causative agent of the disease is buffalopox virus (BPV), which is closely related to Vaccinia Virus (VV) and belongs to the genus *Orthopoxvirus* of the subfamily *Chordopoxvirinae* in the family *Poxviridae*[1]. The disease is clinically characterized by localized lesions on udder, teats, inside of thighs, on the base of ears, over parotid region, inner surface of ear flap and eyes in mild form[2] while in the severe form, the lesions are generalized[3,4].

Buffalopox is one of the important viral zoonotic diseases and is frequent in countries where buffaloes are reared as milch animals[5]. In the past, buffalopox outbreaks have occurred frequently and consistently in various states of the country viz. Haryana, Maharashtra, Uttar Pradesh, Andhra Pradesh, Madhya Pradesh and Karnataka[6-8] besides Pakistan, Bangladesh and other neighboring countries[2].

Development of prophylactics and diagnostics against buffalopox is highly imperative, since the disease is of zoonotic importance and the virus as well as BPV antibodies have been detected in human population also[9,10]. In the absence of smallpox vaccination in human beings since its eradication in 1980, cross infection from animal poxviruses like buffalopox and cowpox creates a significant risk for human beings. The risk of contacting pox-like disease by humans is thus a serious issue that needs to be addressed by developing disease control measures against buffalopox that may otherwise constitute a further risk of pox epidemics in humans in the future. The presence of disease in India makes it as a focus for spread of the disease to other parts of the world and poses a serious threat of pox-like disease in buffaloes and humans.

The BPV has not been studied in detail, as the disease is mainly prevalent in Indian sub-continent. Characterization of the virological and immunological properties of the BPV isolates from various geographical

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areas of the country is a prerequisite for the development of suitable immunodiagnostics and immunoprophylactics in future. To investigate the possible variations in polypeptide composition and study their immunogenic property, the present study was undertaken with an aim of isolation of BPV from suspected clinical samples, adaptation in Vero cells and purification of cell culture grown viruses by density gradient method in order to characterize the viral polypeptides from field isolate as well as BP4 reference strain.

MATERIALS AND METHODS

Viruses: Details of field isolates of BPV from different geographical areas of India including their origin and the year of isolation are listed in Table 1. The reference virus (BP4) virus was originally isolated in the laboratory of Department of Bacteriology and Hygiene, Haryana Agricultural University, Hisar (India)[6]. All the isolates listed are available in the repository of the Poxvirus Laboratory of Division of Virology at IVRI, Mukteswar.

Screening of field clinical samples by PCR: Field specimens in the form of scab materials either submitted to the lab for diagnosis or collected during outbreaks were initially subjected to PCR based on ATI (A type inclusion body) gene of orthopoxvirus (OPV). Total viral DNA was extracted directly from scab materials using AuPreP™ GEN^{bt} DNA Extraction Kit (AuPreP™ Life Technologies (India) Pvt. Ltd, India) as per manufacturer's protocol. The extracted DNA was subjected to PCR amplification using virus specific CoPV3 (5'd AGGGATATCAAGGAAT GCGA-OH3') and CoPV4 (5'd TCCATATCAGCATTGCTTTC-OH3') as upstream and downstream primers, respectively to amplify partial sequences of ATI gene of BPV. These primers were designed based on the published cowpox virus sequence data[11,12].

The PCR reaction was carried out in 50 μL reaction mix containing 2mM $MgCl_2$, 100 μM each of the four dNTPs, 25 mM Tris HCl, 50mM KCl, 10 pmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen life technologies, Carlsbad, CA, USA) using a thermal cycler (PTC 200, MJ Research, USA). The PCR cycling conditions included initial denaturation at 95° C for 5 min followed by 35 cycles of 94°C for 30sec, 55°C for 30 sec and extension 72°C for 30sec with a final extension of one cycle for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel along with known molecular weight DNA (100bp DNA ladder size) marker to confirm the 552bp size of the products and documented.

Table 1: Details of BPV isolates recovered from field clinical samples

Virus isolates	Source	Place and year of isolation
BPV-BP4	Reference virus	Hisar, HAU[6]
BPV-Vij 96	Scab	Vijayawada,AP 1996
BPV-Vij 97	Scab	Vijayawada,AP 1997
BPV-Bly	Scab	Bareilly, UP, 1999
BPV-1999	Scab	Bareilly, UP, 1999
BPV-Hyd	Scab	Hyderabad, AP, 2003
BPV-Aur	Milk /Scab	Augrangabad, MS, 2003
BPV-Pune	Milk/Scab	Pune, MS, 2003
BPV-Bang	Scab	Bangalore, Karnataka, 2004

Virus isolation: The PCR positive field skin scab materials were ground finely with sterile sand as 10% (w/v) suspensions in PBS, clarified by centrifugation at 3000xg for 5 min, treated with antibiotics (Gentamicin) and incubated at 4°C for 1 h and then filtered through 0.45 micron membrane filter. The filtrate (0.5 to 1 mL) was inoculated onto preformed Vero cell monolayer in 25cm^2 flask. Monolayers were incubated at 37°C for 1 h under intermittent shaking to allow adsorption of virus, after which the unadsorbed virus was decanted, monolayer washed with medium and fed with EMEM (Eagle's Minimal Essential Medium) containing 2% Faetal Bovine Serum (FBS). Sometimes, one or two blind passages of the infected cells were necessary to recover the virus. Monolayers were maintained for 5 days and observed daily for cytopathic effects (CPE- degeneration and rounding of cells, micro plaque formation and detachment of cell monolayer at 72 hrs of infection).

Adaptation of virus isolates: The isolates showing CPE characteristic of BPV were further passaged, titrated and stored for further use. Titration of the BPV virus was carried out in Vero cell monolayer following standard protocol and titre was calculated by using Reed and Muench formula[13] and expressed as log tissue culture infective dose 50% ($TCID_{50}$).

Bulk cultivation of virus: Vero monolayer cells were grown in 1700cm^2 roller flasks (Greiner, Denmark) in 200 mL growth medium (EMEM with 10% FBS) and used for the production of virus. The Vero cell adapted BPV field virus suspension @ 0.01 to 0.1 m.o.i (multiplicity of infection) was inoculated to the monolayer cells and allowed for adsorption at 37°C for 1 h. Then the monolayers were fed with maintenance medium (EMEM with 2% FBS) and incubated further for 3-4 days till ≥ 80% CPE was observed. Infected cell cultures showing ≥80% CPE were harvested and subjected to three cycles of freezing and thawing and stored at-20°C until further use Purification of BPV: Purification of BPV using sucrose density gradient was carried out by following the method of Kitching *et al.*,[14] with some modifications. The harvested supernatant was subjected to clarification at

6000xg for 30 min at 4°C. Clarified infected fluid was precipitated using 8% (W/V) PEG 8000 following standard protocol, then centrifuged at 8500x g for 30 min and finally the pellet was resuspended ($1/10^{th}$ of the original volume) in TNE buffer (10mM tris, 150mM NaCl, 1mM EDTA, pH 8). The resulting partially purified antigen was layered over 36% sucrose cushion and centrifuged at 82500xg for 90 min in an ultracentrifuge (OTD-65B Sorvall DuPont, USA). Healthy Vero cell culture was treated similarly as control. The pellet after sucrose cushion was ultrasonicated and layered over Sucrose (Sigma Aldrich Chemical corporation, St. Louis, USA) discontinuous gradient 60-36% and centrifuged at 57640xg for 90 min in a swing out rotor (AH 629 rotor, Sorvall® DuPont, USA). The opalescent viral band at the interface and band over 36% sucrose layer were collected using Pasteur pipette and pelleted at 82000xg for 1 h at 4°C and resuspended in TNE buffer.

Assessing the purity of virus: The absorbance values of the purified virus preparation were taken at 260 nm and 280nm in a UV spectrophotometer (Thermospectronic, Biomate, England). The approximate viral protein concentration and purity was assessed by UV Spectrophotometry method using the formula: [a] Protein Concentration (mg mL^{-1}) = O.D.280x1.55-O.D. 260x 0.77), and [b] Purity = ratio of 260/280 O.D, respectively as per the standard method[15]. The virus preparations giving ratios of 1.2 and above were considered as purified. Purity was also verified by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and virus infectivity assay in cell culture. Purified BPV isolates along with reference BP4 virus were subjected to discontinuous 12% (SDS-PAGE) as per the method of Laemmli[16]. The purified viruses (BP4 and Vij 97 isolates) were diluted 1:10 concentration in TE buffer, filtered through 0.2 micron membrane filter and titrated in Vero cells. The titrated purified virus in 100 µl volume (0.1 m.o.i) was subjected to infectivity assay.

RESULTS AND DISCUSSION

There have been a few reports on the isolation of the BPV from Pakistan, Egypt, Italy and some other countries. The work on virological and serological characterization[17] and molecular characterization employing restriction profiling[18] related to BPV isolates originating from India have been published. Isolation of virus in cell culture, collection of field serum and the virus isolates in the repository are the prime requirements towards the virological, serological and molecular analysis of the causative agent of a particular disease. This helps in generating the base line data in relation to the disease and its causative agent, which may, in turn, help in developing suitable diagnostics and prophylactics.

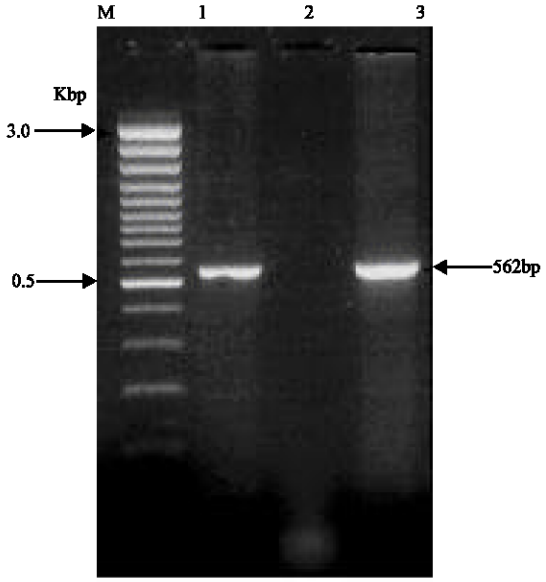

Fig.1: Agarose gel electrophoresis of PCR products based on fragment of ATI gene of virus
Lane M- 100 bp Ladder plus (MBI)
Lane 1- Positive control (BP4)
Lane 2- Negative control
Lane 3 -BPV/Aurangabad (Aur04)

In this study, we attempted the isolation of BPV from suspected clinical samples from different outbreaks of country, their adaptation in Vero cell line, and purification of these BPV isolates by density gradient method with a futuristic viewpoint to characterize BPVs prevalent in various geographical areas and to study their virological and immunological properties.

Field samples either submitted to the lab for diagnosis or collected during outbreaks were initially subjected to PCR amplifying inclusion gene of BPV using the CoPV-specific primers. The DNA extracted from scab materials was used as template for PCR (Fig. 1) using CoPV-specific primers[11,12]. The PCR-positive clinical field samples were further passaged in Vero cells for virus isolation and subsequent adaptation to Vero cells. Vero cell adapted BPV showed characteristic CPE viz., degeneration and rounding of cells, micro plaque formation and detachment of cell monolayer at 72 h of infection (Fig. 2). The cell culture-adapted virus was titrated at different passage level to assess virus amount. The results pertaining to virus titre values of various isolates of BPV are presented in Table 2. The Vero cell adapted BPV isolates giving highest titre at a

Fig. 2: Vero cell line infected with BPV showing cytopathic effects- degeneration and rounding of cells, micro plaque formation and detachment of cell monolayer at 72 hrs of infection (Formaldehyde fixation with crystal violet stain)

Table 2: BPV isolates at different passage level and its titration in Vero cell line

Virus Isolates	Passage level	Titre TCID50 mL^{-1}
BPV-BP4	49	7.55
BPV-Vij 96	12	6.55
BPV-Vij 97	5	6.05
BPV-Bly	7	5.75
BPV-1999	9	6.55
BPV-Hyd	3	5.45
BPV-Aur	3	5.25
BPV-Pune	3	5.55
BPV-Bang	4	5.67

particular passage level were used further for bulk production of virus.

BPV was purified using discontinuous (60-36%) sucrose density gradient and the virus banded at the interface of two layers of sucrose where as the colored contaminants and cell debris settled as pellet and also remained in upper gradient of sucrose solution. However, we observed two white opalescent bands after the gradient ultracentifugation (Fig. 3); the upper band above the 36% sucrose density being the Extracellular Enveloped Virus (EEV) having less density, where as the opalescent band between the density gradient is the pure naked intracellular mature virus (IMV) and has high density than the envelope virus. To characterize these two components
of BPV and assess the purity, the infectivity assay in cell culture was carried out.

The virus bands obtained were checked for their purity by taking OD 260/280 ratio. The viral protein

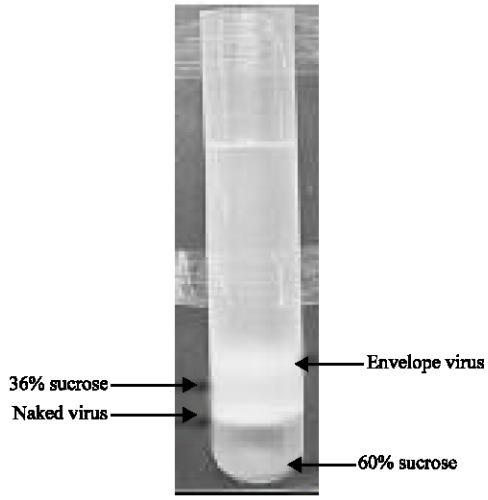

Fig.3. Sucrose gradient purification of buffalo poxvirus

concentration and purity of reference virus was measured by UV Spectrophotometry and found to be 6.36 mg mL^{-1} and 1.4, respectively. Similarly, the extinction ratio values (O.D. 260/280) for the all the BPV isolates were within the range of 1.2 to 1.4, indicating the highly purified nature of the BPV. The viral polypeptides of BPV isolates and reference virus were identified and characterized by SDS-PAGE. On the basis of polypeptide composition of BPV isolates and BP4 virus examined with 12% SDS-PAGE, all three BPV isolates were similar and contained more than 25 polypeptides with a molecular weight range from 14.2 kDa to 180 kDa (Fig. 4) which is in consonance with the reported data. Singh[19] and Maan and Kalra,[20] described polypeptide analysis after SDS-PAGE using purified virus preparations. The analysis of the polypeptide profile of BPBB (buffalo isolate), BPBH (human isolate) and Vaccinia Viruses (WR strain) by SDS-PAGE revealed 26, 19 and 29 polypeptides and 6, 3 and 7 glycoproteins, respectively. However, the number of polypeptides observed did not give clue to relatedness of viruses studied warranting therefore further study on protein profiling of the BPV.

To confirm the envelope nature of upper virus band, the infectivity assay was carried out with both the opalescent bands. Infectivity assay of the purified virus isolates namely BP4 (Fig. 5) and Vij 97 in Vero cells revealed the titres of $10^{-4.25}$ $TCID_{50}$/ ml and$10^{-3.50}$ $TCID_{50}$/ ml for BPV-BP4 enveloped and naked virus, respectively while the titre of BPV- Vij 97-envelope and naked virus was found to be $10^{-3.50}$ $TCID_{50}$/ mL. The envelope virus showed high infectivity and titre compared to naked virus even with the same titre of both the purified bands used in the infectivity assay. The high infectivity of envelope virus has also been reported for other poxviruses[21].

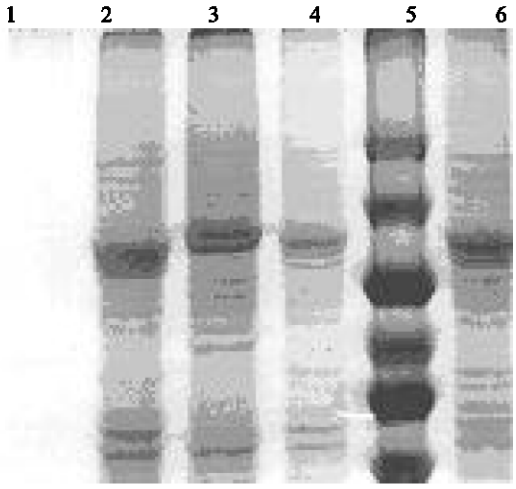

Fig 4: Protein profile of purified BPV isolates by SDS-PAGE
Viral proteins from different isolates
Lane-1 Cell Control
Lane-2 Vijayawada 1996
Lane-3 Vijayawada 1997
Lane-4 Bareilly
Lane-5 Protein molecular weight marker (Promega)
Lane-6 BPV-1999

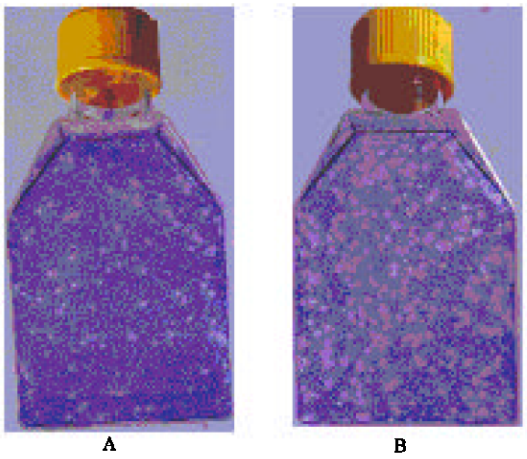

Fig. 5: Infectivity Assay of purified BPV in Vero cells Cytopathic changes in Vero cells produced by envelope virus at 4th day p.i (A) and naked virus at 5th day p. i. (B)

CONCLUSION

Indian isolates of BPV from field clinical samples were isolated, adapted in Vero cell culture and purified using sucrose density gradient, which will help in undertaking further work on protein profile and its characterization.

Further, the reference strain (BP4) and field isolate were similar in their protein profile and infectivity in Vero cells. This data will serve as baseline data for further characterization of the virological and immunological properties of BPV field strains in order to develop suitable immunodiagnostics and prophylactics.

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