

**QPCR BASED POTENCY ESTIMATION AND
THERMO STABILITY EVALUATION OF LIVE
ATTENUATED BUFFALO POX VACCINE**



Thesis

Submitted in partial fulfilment of the requirement for the degree

of

MASTER OF VETERINARY SCIENCE

in

VETERINARY VIROLOGY

By

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To

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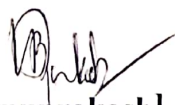
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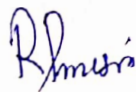
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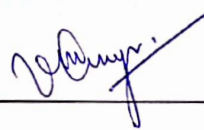
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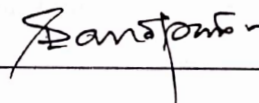
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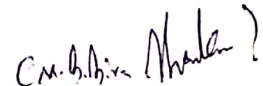
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Date: 21.5.2016
Place: Mukteshwar


M.S. Siva Shankar

Abbreviations

μl	microlitre
BLAST	Basic local alignment search tool
bp	base pair
BPXV	buffalopox virus
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EMEM	Eagles Minimum Essential medium
Fig	Figure
g	Gravitational force
IPTG	Isopropyl- β-D-thio-galactopyranoside
ml	milliliter
mM	millimole
ORF	Open reading frame
PCR	Polymerase chain reaction
pmol	picomole
QPCR	quantitative polymerase chain reaction
rpm	revolutions per minute
Ta	annealing temperature
TAE	Tris acetate EDTA
UV	Ultraviolet

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Livestock sector is an important sub-sector of Indian agriculture and plays a major role in Indian economy of which buffaloes comprises an integral component and provides livelihood to rural population. In India, buffaloes are mainly reared for both milk and meat production. With the advancement and adoption of scientific managerial practices, the buffalo husbandry industry in India has made considerable progress in the recent years. However, at the same time, many emerging problems have interrupted its speedy development. Incidence of various infectious diseases is one of the most important problems faced by the farmers, of which viral diseases are commonest one. Buffalopox is such a contagious viral disease affecting buffaloes (*Bubalus bubalis*) and rarely cows with morbidity of 80% causing high economic loss to the farmers.

The first recorded incidence of buffalopox dates back to 1934 and occurred in Lahore in undivided India (Sharma, 1934). This was followed by regular reports of both mild and severe forms of the disease from Maharashtra only (Sehgal *et al.*, 1977). The causative agent of buffalopox, buffalopox virus (BPXV), is classified in the *Orthopoxvirus* (OPV) genus of the subfamily *Chordopoxvirinae* and the family *Poxviridae* (Van Regenmortel *et al.*, 2000).

Clinically, buffalopox occurs in mild as well as severe form. In the mild form, lesions are localized on the udder, teats and inguinal region (Sharma, 1934), over the parotid, and the base and inner surface of the ear and eyes (Bhatia, 1936; Wariyar, 1937; Mallick and Dwivedi, 1982; Mallick, 1988). In the severe form, the lesions are generalized (Ramakrishna and Ananthapadmanabham, 1957; Chandra *et al.*, 1987). However, generalized forms of the disease are infrequent these days as the lesions are mostly confined to the udder, teats, and

sometimes on the thighs and hindquarters of the affected animals. Infection in milk animals leads to mastitis, which contributes to reduction in milk yield and the working capacity of draft animals. In severe cases of mastitis, a permanent reduction in milk yield is also recorded (Singh *et al.*, 2006c). Although buffaloes are primarily affected, cows and humans are occasionally involved (Baxby and Hill, 1969). Outbreaks of buffalopox are reported not only from India but also from many other countries including Pakistan, Egypt, Nepal and Bangladesh (Lal and Singh, 1977). Recently, similar outbreaks of pox-like-infections caused by Vaccinia virus (VACV)-like agents viz. Cantagalo (Damaso *et al.*, 2000) and Aracatuba viruses (de Souza Trindade *et al.*, 2003) have been reported from Brazil.

Of late, buffalopox has been recognized as an important emerging and re-emerging zoonosis (Singh *et al.*, 2007). The Joint Expert Committee on Zoonosis also declared buffalopox as one of the important zoonotic diseases. The committee also emphasized that the mode of transmission of buffalopox to human subjects and other epidemiological features appear similar to cowpox (Chandra *et al.*, 1987). Human beings including smallpox-vaccinees contract infection upon close contact with buffalopox virusinfected animals, but the extent of disease is greater in non-immunized individuals. The appearance of gross lesions in human beings maintaining close proximity with pox-affected buffaloes, attendants and milkers has been reported from time to time (Wariyar, 1937; Ghosh *et al.*, 1977; Mehrotra *et al.*, 1981; Mitra and Chatterji, 1986; Kumar *et al.*, 1987; Kolhapure *et al.*, 1997; Raut *et al.*, 1997; Jain, 1998; Singh *et al.*, 1999, 2006c, 2007; Bhanuprakash *et al.*, 2009). The lesions are mainly confined to the hands, forehead, face, buttocks and legs. Occasionally, lymphadenopathy has also been recorded. Human-to-human transmission has not been reported. Until recently, BPXV was considered host specific infecting only buffaloes but experiments on biological transmission of BPXV (BP4 strain) indicated that BPXV could be transmitted to cow calves if given in a high dose (Singh *et al.*, 1996).

Control of any infection depends on accurate diagnosis and treatment/prophylaxis. Accordingly, buffalopox can easily be diagnosed by clinical signs. However some times clinical lesions can confuse with other viral diseases such as papilloma, foot and mouth disease (FMD) and capripox, hence laboratory confirmation is necessary. The laboratory tests include virus isolation, electron microscopy, histopathology of the affected tissues, specific serological assays

viz., enzyme linked immunosorbent assay (ELISA), serum neutralization (SNT) and nucleic acid based methods such as polymerase chain reaction (PCR) and real-time PCR.

In countries where the disease is endemic and animal movement is difficult to restrict, disease control becomes complex. The problem is compounded by lack of precise diagnostics and prophylactics. Further, the contamination of buffalo meat for export is another problem. The social belief, cultural issues and economic considerations prevent India from having a slaughtering policy for cattle. In the absence of suitable immunoprophylaxis and restriction on animal movement, employing sanitary measures such as segregation of the infected animals is the only means of containing the spread of infection. In our laboratory, recently a live attenuated vaccine was prepared using Vero cell-adapted BPXV (VIJ96) and compared (unpublished data) with the already available reference strain (BP4) vaccine. It was found that the BP4 vaccine was still superior in terms of potency compared with the Vero cell adapted vaccine.

Further, India being a subtropical country, it experiences wide range of temperatures. Therefore, maintenance of shelf-life of the vaccine is very difficult and needs cold chain. Like many other modified live attenuated lyophilized vaccines, the attenuated buffalopox vaccine is no exception in thermal inactivation. The vaccine requires cold chain for storage and transport. As a result, the cost of production and transportation are high and user's expenses are unavoidable, especially in developing tropical and semitropical countries like India. Therefore, evaluation of the vaccines for their thermo-stability with different stabilizers is of very much demanding in order to maintain or improve immunization effectiveness through reduction of potency loss due to heat damage; reduce cold chain costs and logistical requirements.

Likewise, the efficacy (sero-conversion) and safety of live attenuated vaccine depends on the number of viable particles in the vaccine dose. One of the steps in quality control of vaccine is to fix the number of live viral particles per dose, which varies with the type of vaccine as prescribed by the manufacturers. To estimate the number of infectious viral particles in the live attenuated vaccine, commonly used assays include, tissue culture infective dose (TCID₅₀) or Plaque forming unit (PFU) in cell culture, or egg infective dose (EID₅₀) estimation in embryonated eggs, respectively. However, these assays are time consuming and labor intensive, require extensive standardization and interpretation of results is mainly subjective. Quantitative polymerase chain reaction (QPCR) has presently been used as novel approach

for the potency estimation of multivalent human vaccines (Schalk *et al.*, 2004; Ranheim *et al.*, 2006), animal vaccines like goatpox and PPR (Kallesh *et al.*, 2009) for its advantages such as high sensitivity, specificity, reproducibility and rapidity. The QPCR is very flexible and need no post PCR manipulations. Identification of the amplified product is very specific as detection chemistries used in the QPCR are highly specific. The commonly used ones are SYBR green I dye and TaqMan probes. In view of these, the present work has been designed with the following objectives;

- **To evaluate the thermo-stability and potency of live attenuated Vero cell adapted buffalopox vaccine.**



History of Buffalopox

The first recorded incidence of buffalopox dates back to 1934 and occurred in Lahore in undivided India (Sharma, 1934) and the term ‘ buffalopox’ was first used by Haddow and Idnani(1949).This was followed by regular reports of both mild and severe forms of the disease from various states of India (Sehgal *et al.*, 1977). Outbreaks of buffalopox are reported not only from India but also from many other countries including Pakistan, Egypt, Nepal and Bangladesh. Recently, similar outbreaks of pox-like-infections caused by Vaccinia virus (VACV)-like agents viz. Cantagalo (Damaso *et al.*, 2000) and Aracatuba viruses (de Souza Trindade *et al.*, 2003) have been reported from Brazil. The WHO Joint Expert Committee on Zoonosis (FAO, 1967) has declared buffalopox to be one of the important zoonotic diseases caused by a virus, closely related to VACV and frequently encountered in countries where buffaloes are reared as milch animals.

Etiology

The causative agent of buffalopox, buffalopox virus (BPXV), is classified in the *Orthopoxvirus* (OPV) genus of the subfamily *Chordopoxvirinae* and the family *Poxviridae* (Regenmortel *et al.*, 2000). It is a double stranded DNA virus with a genome of approx. 130-200 Kb (Murphy *et al.*, 1999). BPXV resembles vaccinia and cowpox viruses (Lal and Singh, 1977).The BP4 strain (Department of Bacteriology and Hygiene, Haryana Agricultural University, Hisar, India) is regarded as the reference strain of the virus (Singh and Singh, 1967; Baxby and Hill, 1969).

Epidemiology

After the first report of buffalopox in 1934, disease has been regularly reported from buffalo-rearing areas involving different states of India. The disease has mainly been recorded in young and old buffaloes during the epidemics. However, cattle have also been occasionally affected (Ghosh *et al.*, 1977). Transmissibility of BPXV was experimentally studied (Singh *et al.*, 1996) in a number of animal species, which revealed a wide host range, viz. buffaloes, cows at high dose of virus, guinea pigs and suckling mice (BALB/c and Swiss white strains). Strain BPH-80 produced pox lesions in rabbits, 6-weekold mice, cow and buffalo calves, and chicken. Sheep, goat, fowl and adult mice (BALB/c and Swiss white) were refractory to experimental BPXV infection (Kumar *et al.*, 1987). An overall disease prevalence rate of 10.13% was reported in one study, in which there was no significant difference between prevalence in adult males (13.63%) and females (13%) and young males (6.32%) and females (5.24%) (Kumar *et al.*, 1987). Mastitis due to secondary bacterial infections has been recorded in 50% of affected lactating buffaloes. A higher prevalence rate (23.4–79.4%) was reported in another buffalopox outbreak from Mandya and Mysore (Karnataka; Muraleedharan *et al.*, 1989). A recent outbreak of buffalopox in Aurangabad (Maharashtra) caused a morbidity rate of up to 45% (Singh *et al.*, 2006). Although 5.6% seropositivity in buffaloes has been reported using CFT from various parts of Egypt (Iwad *et al.*, 1981), the spread of disease was rapid in buffaloes but cows, sheeps and goats in the same areas remained unaffected. Biting flies such as *Lyperosia exigua*, *Musca crossirostris* and *Musca vicina* aggravated the sores. Therefore, it was contended that flies may be involved in mechanical transmission (Muraleedharan *et al.*, 1989).

Buffalopox as zoonosis

Buffalopox is an important contagious viral malady of buffaloes of all ages, occurring in epidemic proportions in countries including India, where buffaloes are reared. The Joint Expert Committee on Zoonosis declared buffalopox as one of the important zoonotic diseases. The committee also emphasized that the mode of transmission of buffalopox to human subjects and other epidemiological features appear similar to cowpox (Chandra *et al.*, 1987). Human

beings including smallpox-vaccinees contract infection upon close contact with buffalopox virus infected animals, but the extent of disease is greater in non-immunized individuals. The appearance of gross lesions in human beings maintaining close proximity with pox-affected buffaloes, attendants and milkers has been reported from time to time (Wariyar, 1937; Ghosh *et al.*, 1977; Mehrotra *et al.*, 1981; Mitra and Chatterji, 1986; Kumar *et al.*, 1987; Kolhapure *et al.*, 1997; Raut *et al.*, 1997; Jain, 1998; Singh *et al.*, 1999, 2006). The lesions are mainly confined to the hands, forehead, face, buttocks and legs. Occasionally, lymphadenopathy has also been recorded. Human-to-human transmission has not been reported

Diagnosis

The conventional isolation methods of buffalopox virus (BPXV) include either passaging in chorio-allantoic membrane (CAM) or inoculation of specific hosts – buffalo or cattle. Although various serological assays have been developed for diagnosis of buffalopox including agar gel immuno-diffusion (AGID), counter-immunoelectrophoresis (CIE), serum neutralization test (SNT), enzyme linked immunosorbent assay (ELISA) and immunoperoxidase test (IPT), these tests fail in accurate diagnosis of the disease because of antigenic cross-reactivity. Conventional biological and serological methods have limitations in identification and differentiation of orthopox viruses (OPVs) (Murphy *et al.*, 1999). Analysis of virion polypeptides by SDS-PAGE has enabled genus and species differentiation but not between closely related members of the same genus (Arita and Tagaya, 1977; Esposito *et al.*, 1977). However, RE–DNA profiling (Esposito and Knight, 1985) has been a proven method for poxvirus identification but requires virus propagation and viral genomic DNA isolation, which is time-consuming (Singh *et al.*, 2007).

PCR-based assays have been described for diagnosis and differentiation of OPVs like vaccinia virus (VACV), Variola virus (VARV), monkeypox virus (MPXV) and Camelpox virus (CPXV), based on inclusion and HA genes (Knight *et al.*, 1995; Ropp *et al.*, 1995; Meyer *et al.*, 1997; Damaso *et al.*, 2000)

Prevention and control

In countries where the disease is endemic and animal movement is difficult to restrict, disease control becomes complex. The problem is compounded by lack of precise diagnostics

and prophylactics. Further, the contamination of buffalo meat for export is another problem. The social belief, cultural issues and economic considerations prevent India from having a slaughtering policy for cattle. In the absence of suitable immunoprophylaxis and restriction on animal movement, employing sanitary measures such as segregation of the infected animals is the only means of containing the spread of infection. In our laboratory, recently a live attenuated vaccine has been developed using Vero cell-adapted BPXV (VIJ96) and compared (unpublished data) with the already available reference strain (BP4) vaccine. The vaccine is currently undergoing extensive field trials in different parts of the country.

Thermostability of live attenuated vaccines

India being a subtropical country, it experiences wide range of temperatures. Therefore maintenance of shelf-life of the vaccine is very difficult and needs cold chain. Like many other modified live attenuated lyophilized vaccines, the attenuated buffalopox vaccine is no exception in thermal inactivation. The vaccine requires cold chain for storage and transport. As a result, the cost of production and transportation are high and user's expenses are unavoidable, especially in developing tropical and semitropical countries like India. Therefore, evaluation of the vaccines for their thermo-stability with different stabilizers is of very much demanding in order to maintain or improve immunization effectiveness through reduction of potency loss due to heat damage; reduce cold chain costs and logistical requirements.

There are only few reports regarding thermo stabilization of animal viral vaccines. The notable ones are against Newcastle disease (Bensinki and Spradbrow, 1995) and rinderpest (RP) (Raut *et al.*, 2001). The earliest reports on the thermo-stability of the cell culture adapted RPV were of Plowright and Ferris (1961) and Johnson (1962). Rinderpest virus was selected for mutant clones by growing the virus for 6 passages at 40°C. The increment of temperature was given at intervals of 0.5°C. It was seen that compared to the vaccine virus, thermo-resistant virus multiplied at higher temperatures (40°C). On evaluation of both viruses, it was seen that immunogenic protein profiles are similar, indicating that there was no change with regard to these characteristics but there were minor changes observed in the amino acid sequences on the basis of nucleotide sequence analysis (Raut *et al.*, 2001).

Reports regarding the development of thermo-resistant strains of NDV notably I-2 and V-4 strains from avirulent Australian strains of NDV have been published. These strains were selected on the basis of two criteria viz., ability to produce rapid antibody response and stability at 56°C. These vaccines were used either as ocular or oral drops or incorporation in cooked white rice (Rehmani and Spradbrow, 1995).

A lyophilized thermo-stable Vero cell-adapted RP vaccine, stabilized with lactalbumin hydrolysate (LAH) and sucrose, was tested for safety, serological response and suitability for use with an abbreviated cold-chain under field conditions in Nigeria. Cattle, goats and sheep of unknown serological status were vaccinated at organized ranches and observed for 22 days. No untoward effects were detected. The serological response to the vaccine stored at environmental temperatures for 30 to 34 days was determined in 144 previously unvaccinated yearling calves. Sero-conversion was demonstrated in 98% of the yearling calves using serum neutralization. The un-refrigerated vaccine retained a titer of $3.69 \log_{10} \text{TCID}_{50}/\text{dose}$ through day 34 (Mariner *et al.*, 1990). Based on this approach, recently, the Indian Veterinary Research Institute (IVRI), Mukteswar, have been successful in developing live attenuated Tr-PPR vaccines (goat and sheep isolates) employing thermo-stable Vero cell line which is adapted to grow at 40°C. The vaccines have undergone successful in-house trials both in the sheep and goats either as single or in combination with sheeppox or goatpox as bivalent or Orf as trivalent vaccines (Personal Communication). In an another study conducted by our laboratory, thermostability of high passaged (P100) GTP vaccine was carried out using LAH + 10% Sucrose (LHS), LAH+10% trehalose dihydrate (LHT), Trehalose dihydrate 10% in HBSS (T-10) and Trehalose dihydrate 5% in heavy water (DT-5) stabilizer combinations. Vaccine vials with different stabilizers were lyophilized and exposed at different temperatures viz., 45°C, 37 °C, 25 °C and 4 °C and titrated. LHT found relatively superior than LHS and T-10; Relative difference of stabilization between LHS and T-10 were recorded. No significant loss of virus titre in freeze-dried vial for 5 days at 45 °C; 9 days at 37 °C & 98 days at 4 °C was observed (Personal communication).

Potency estimation of live attenuated Vaccines

Unlike inactivated vaccines, attenuated live vaccines (ALV) require an *in vivo* potency assay on each batch, the whole dose–response curve of modified live vaccine (ALV) is assessed only once on the master seed virus or a representative vaccine batch of the virus seed. The potency of subsequent batches can be based on the relation between virus dose and protection as observed in the dose-response curve of the master seed (Terpstra and Kroese, 1996). One of the important causes of vaccine induced disease outbreak or vaccine failure is excess or inadequate infectious viral particles. Hence in recent days, control authorities made a mandate to have a fixed amount of viral load in their vaccine formulations. The *European Pharmacopoeia* generally requires that one dose of reconstituted vaccine is equivalent to the minimum virus titer stated on the label/ leaflet and that amount satisfies the criterion for potency acceptance of the product. United State code of federal regulation of animals and animal products [US-Code of Federal Regulations (CFR 1993)] has made mandate that batches of ALV should contain at any time within the expiration period a virus titer of $10^{0.7}$ titer greater than the titer of master seed virus that provides an accepted level of protection in animal challenge experiment for potency testing.

From decades back, plaque assay (PFU) and tissue culture infectivity dose (TCID₅₀) estimation were used as standard assays for estimation of the infectivity of the ALV. Eventhough the plaque assay is considered to be the standard assay for estimation of number of infectious viral particles, assay has some serious setbacks. It has been observed that the results of plaque assay are influenced by several parameters including the incubation temperature, virus inocula and pH of the agar used. With rubella vaccine virus, some strains produce higher infective virus titres at lower temperature, while some need higher temperatures to produce desirable plaques. The temperature also influenced the morphology of the plaques produced by individual virus strains (Fukuda *et al.*, 1996). The pH of agar and virus inoculums are the other factors, which affect the accuracy of plaque assay (Umino, 1995; Fukuda *et al.*, 1996).



3.1. MATERIALS

3.1.1. Reagents and Buffers

Detailed formulation of all the reagents and buffers used in this study are given in Appendix.

3.1.2. Cells

Vero cells (African green monkey kidney) obtained from ATCC (CCL81) maintained in the Division of Virology, Indian Veterinary Research Institute (IVRI) campus Mukteswar were used for growth and titration of buffalopox vaccine virus. The cells were propagated in Eagle's minimum essential medium (EMEM) containing 10mM HEPES, 2mM glutamine and sodium bicarbonate supplemented with 10% new born bovine calf serum (NBCS). For maintaining the cells, maintenance medium (MM) containing EMEM with 2% NBCS was used.

3.1.3. Viruses

The seed virus (buffalopox virus isolate, Vij/96, P-49) collected from the scab material of buffalo infected with buffalopox, maintained in the pox virus laboratory was used for preparation of the vaccine. Different batches of live attenuated buffalopox vaccines prepared using different stabilizers for thermo-stability evaluation as well as for estimation of live viral particles by QPCR.

3.1.4. DNA isolation

DNA was extracted using Au Prep Genomic DNA Isolation kit (M/s Life Technologies India (Pvt) Ltd, New Delhi) as per the manufacturer's instructions.

3.1.5. Enzymes

The Taq DNA polymerase enzyme (Invitrogen) was employed in the study for performing conventional as well as real time PCR assay. T₄ DNA ligase (M/s MBI Fermentas, Maryland, USA) was also used in the study.

3.1.6. Oligonucleotide primers

Details of the oligonucleotide primers used in the study are given in Table 1. The primers used were previously reported and were got synthesized from *Metabion*, Planegg-Martinsried, Germany.

Table 1: Primers used in PCR and QPCR

Name of Primer	Orientation	Sequence (5'-3')	Length	Gene target	Reference
BPXV-C18L F	Forward	CGGTTATTGGAATATGGAGCGAGTG	25	C18L (Truncated Ankyrin repeat protein)	Balamurugan <i>et al.</i> , 2008
BPXV C18L R	Reverse	CGTAGTAATCGTCGTAGGGAGAGAC	25		
Probe(P1)	Forward	HEX-CGTAGTAATCGTCGTAGGGA GAGAC-BHQ1	25		
CoPV 3	Forward	AGGGATATCAAGGAATGCGA	20	ATI gene (A type inclusion)	Meyer and Rziha, 1993)
CoPV 4	Reverse	TCCATATCAGCATTGCTTTC	20		

3.1.7. Kits

- Genomic DNA extraction kit- AuPrepTMGEN^{bt}DNA Extraction kit [M/s Life Technologies India (Pvt) Ltd, New Delhi, India]
- Min Elute Gel Extraction kit: QIAGEN [QIAGEN GmbH, D-40724 Hilden, Germany]
- PCR purification kit- AuPrepTMPCR purification kit [Life Technologies India (Pvt) Ltd, New Delhi, India]

3.1.8. Equipment

i. Microscope

Inverted binocular microscope NIKON –Model TMS (JAPAN)

ii. Centrifuges

- a. Refrigerated microcentrifuge-Biofuge (Heraeus Instruments, Hanau, Germany)
- b. Microcentrifuge-Minispin (Eppendorf AG, Hamburg, Germany)
- iii. Real time PCR system (Model Mx3000P) (Stratagene, La Jolla, CA USA)

iv. PCR Thermal Cycler

- a. Master cycler gradient (Eppendorf AG, Humberg, Germany)
- b. Personal cycler (Eppendorf AG, Humberg, Germany)

v. Incubator

Sanyo CO₂ incubator-MC0175 (Sanyo Electric Co. Ltd., Japan)

vi. Other Equipment

Vortex mixer, cyclomixer (Remi Instruments Pvt. Ltd., Mumbai, India)

Micropipettes of various volumes (0.5µl-1000µl) (Eppendorf India Pvt Ltd)

Horizontal electrophoresis apparatus: Horizon 58 (Gibco BRL, New York, USA)

Orbital Shaker CLA881C C24 (New Brunswick Scientific Co. Inc, Edison, USA)

Gel Documentation system-ImageMaster®VDS (Amersham Biosciences AB, Uppsala, Sweden)

Freezer (-80°C) (Thermo, USA)

Weighing Balance- BP-121S (Sartorius, Germany).

Horizontal and Vertical Laminar Air Flow Cabinets (Klenzaid, India)

Ultracentrifuge (Sorvall, USA). Waterbath (Jouan, France).

3.1.9. Plasticware and glassware

All the plasticware used in the study were procured from M/s Axygen Scientific Inc., Union City, CA, USA; while glassware was procured from M/s Borosil India Pvt Ltd, New Delhi, India and M/s Schott Duran, AG Mainz, Germany.

3.2 METHODS

3.2.1. Revival and bulk production of buffalopox virus

Bulk production of buffalopox virus was carried out in confluent Vero cell line monolayer maintained in roller

The procedure is as follows

- i. One ml of vaccine virus from P 59 passage reconstituted in serum-free EMEM was inoculated on to confluent Vero cell monolayer in a roller (multiplicity of infection approx. 0.01) followed by addition of 100ml of EMEM medium having 2% NBCS. The cells were monitored everyday and the media was changed on every other day till CPE appeared.
- ii. The roller was harvested by repeated freezethawing after 80% CPE appeared, which generally took 24-48 hours.
- iii. To maintain a uniform virus titer, virus harvest from all the roller bottles were pooled after first thaw. The bottles were kept at -80°C until used.

3.2.2. Test for identity of buffalopox vaccine virus

The presence of buffalopox vaccine strains in the culture was confirmed by the following tests:

A. Cytopathic effect (CPE)

All the vaccine viruses infected and mock infected cultures were observed daily under low power and high power inverted microscope for the appearance of buffalopox virus specific CPE.

B. Nucleic acid based detection

The vaccine virus was confirmed by diagnostic PCR (OPV specific) and real-time PCR amplification of viral DNA using specific primers and probe.

C. Cloning and sequencing of selected gene

For further confirmation of identity of the vaccine preparation, the C18L gene was cloned into pGEMT^{easy} vector and sequenced.

3.2.2.1 Density gradient purification of buffalo poxvirus

Sucrose gradient purification of BPXV was carried out to concentrate and purify the BPXV.

- i. The harvested sample was subjected to centrifugation at 6000 rpm for 10 min and supernatant was collected into a fresh container.
- ii. The supernatant was added with PEG 6000 (Poly ethylene glycol, SRL, Bombay, India) at the rate of 8.0% (w/v) and NaCl at 2.3% and then subjected to constant mixing under magnetic stirrer at 4°C overnight.
- iii. Sample was subjected to centrifugation at 6000 rpm for 30 min and pellet was collected.
- iv. The pellet was reconstituted in 5 ml 1X TNE buffer (10mM) and subjected for homogenization @7000 rpm for 4 min.
- v. The sample was overlaid on to 34% sucrose followed by ultra centrifugation at 85000xg for 1 hour. The resultant pellet was collected and again centrifuged over 60% and 36% sucrose gradient and run the ultracentrifuge at the rate of 80000xg for 1 hour. The virus present in the translucent layer interfacing the 64 and 36% layers was collected and pelleted after diluting in TNE buffer.
- vi. The pellet was again collected and resuspended in 1X TNE buffer and store at -80°C till use.

3.2.3. DNA extraction

DNA extraction was carried out using Genomic DNA extraction kit- AuPrep GEN DNA Extraction kit [M/s Life Technologies India (Pvt) Ltd, New Delhi, India]. This method was used to extract the DNA from the purified virus and virus-infected cells as described below:

- i. 20µl Proteinase K and 200l EX Buffer were added to 200µl of purified virus or cell culture harvest in a 1.5 ml microcentrifuge tube and was incubated at 56°C for 1 h after mixing the solution by inverting followed by further incubation at 60°C for 20 min then 70°C for 20 min.
- ii. 210 µl absolute ethanol was added to the solution and was mixed by inverting gently.
- iii. Mixed solution was loaded onto B/T Genomic DNA Mini column (supplied with the kit). With the spin column inside a 2ml collecting tube (supplied with the kit), the column was centrifuged at 9,300 xg for 60 sec at room temperature. Flow-through

was discarded.

- iv. The column was then placed in a clean 2ml collection tube and 400µl of wash buffer I (supplied with the kit) was added. The column was centrifuged at 6,000 xg for 2 min and flow-through was discarded.
- v. The column was then placed in the same collection tube and 500µl DNA WS buffer was added. The column was then centrifuged at 6,000 xg for 2 min and the flow-through was discarded.
- vi. The column was again washed second time with 500µl of DNA WS buffer.
- vii. After discarding the flow-through, the spin cartridge was centrifuged for 1 min at full speed to completely dry the matrix.
- viii. The dried column was then transferred to a new 1.5ml microcentrifuge tube and the DNA was eluted with 50 µl of pre-warmed TE buffer by centrifuging at 10,000 xg for 2 min. Stored at -80°C till use.

3.2.4 Polymerase Chain Reaction (PCR)

The PCR reaction was carried out in standard 50µl reaction for DNA Polymerase gene target following standard protocols. A typical PCR reaction mixture contained the following reagents (Invitrogen, Carlsbad, California, USA) and was performed in a 0.2ml PCR tubes.

Component (stock)	Amount (volume)
10X PCR Buffer	5.0 µl
50mM MgCl ₂	2.0 µl
10mM dNTPs	1.0 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10pmol)	1.0 µl
DNA	2.0 µl
<i>Taq</i> DNA Polymerase (5U/µl)	0.2 µl
Nuclease free water	37.8 µl
Total reaction volume	50.0 µl

The contents were mixed thoroughly and spin briefly. The tubes were then placed in a thermocycler. PCR conditions for the amplification of the target gene using primers specific for orthopoxvirus (ATI-F/ATI-R) and buffalopox virus (C18L-F/ C18L-R) are mentioned in Table 2.

Table 2 Thermal conditions used for conventional PCR

Primers used	Thermal Profile						Expected size of amplicons
	1	2	3	4	5	6	
CoPV 3/4	95 ⁰ C 4mins	94 ⁰ C 1min	52 ⁰ C 1 min	72 ⁰ C 1min	Step2-4 35cycles	72 ⁰ C 7mins	552 bp
C18L F/R	95 ⁰ C 4mins	94 ⁰ C 30 sec	63 ⁰ C 30 sec	72 ⁰ C 45 sec	Step 2-4 35cycles	72 ⁰ C 10mins	138 bp

3.2.5 Agarose gel electrophoresis of Amplicons

1. 2% molten agarose (Sigma-Aldrich, St. Louis, USA) was prepared in 1X TAE buffer (Appendix) in microwave oven.
2. It was then allowed to cool to 50°C and ethidium bromide (10µg /ml) was added to a final concentration of 0.5 µg/ml. About 50 ml of molten gel was poured into horizontal gel electrophoresis apparatus and then comb was inserted. Gel was allowed to solidify at room temperature for 20 min.
3. Electrophoresis tank was filled with 1X TAE buffer and the gel was immersed in buffer and combs were removed.
4. Samples were prepared by mixing 5 µl of PCR product and 1 µl of 6X loading buffer (Sigma-Aldrich, St. Louis, USA) containing 0.25% w/v bromophenol blue, 0.25% w/v, Xylene cyanol FF and 40% w/v sucrose in water.
5. Samples were loaded in parallel with 100 bp molecular weight marker or PCR marker (MBI Farmentas, Maryland, USA). The electrophoresis was carried out for 45 minutes at 70 Volts. Then the gel was visualized in a transilluminator (Amersham Biosciences AB, Uppsala, Sweden) and documented.

3.2.6. Purification of PCR products

Min Elute Gel Extraction kit: QIAGEN [QIAGEN GmbH, Hilden, Germany]. The procedure used was as follows:

- i. The PCR product was run in 1% low melting point (LMP) agarose (Sigma-Aldrich, St. Louis, USA) gel in 1X TAE buffer along with molecular weight DNA ladder.
- ii. With a clean, sharp scalpel or razor blade; the gel slice containing the desired DNA fragment was excised on a UV Transilluminator with minimum UV exposure.
- iii. The slice was then cut into small pieces and was placed into a sterile 1.5 ml microcentrifuge tube.
- iv. 0.6ml QG buffer (supplied with the kit) was added to it and incubated at 50°C in a heated waterbath for 10 min until the gel was completely dissolved. The tube was inverted at every 1-2 min during incubation.
- v. A MinElute column (supplied with the kit) was placed onto a 2ml collection tube provided in the kit and 0.7ml of dissolved gel mixture was added into the column.
- vi. The column was centrifuged at 12,000 xg for 30-60 sec and the flow-through was discarded. Step V was repeated for rest of the mixture.
- vii. The column was washed with 0.7 ml PE buffer (supplied with the kit) by centrifuging for 30-60 sec at 12,000 xg and the flow-through was discarded.
- viii. The column was then centrifuged at full speed for another 3 min to remove residual ethanol present in column.
- ix. The column was placed onto a new 1.5 ml microcentrifuge tube and 12µl of pre-heated elution buffer (provided with the kit) or nuclease free water was added onto the center of the resin column.
- x. The column was left to stand at 37°C for 1-2 min and then centrifuged at 10000 RPM for 1-2 min to elute the DNA. The eluted DNA was stored at -20°C for further use.

3.2.7. Cloning of the PCR product

The purified PCR products were cloned into pGEM-T Easy vector using TA cloning mechanism (Promega, WI, USA). The PCR products were quantified in 1% agarose gel by densitometric comparison. The appropriate amount (ng) of the PCR product was subsequently cloned into the plasmid vector keeping the insert: vector molar ratio to 3:1 as per the following calculation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert (PCR product)}$$

a. Ligation of the PCR Products into the Plasmid Vector

The PCR products were ligated with desired vector as mentioned below

Purified PCR product	2-5µl	(depending on product concentration)
pGEM-T Easy Vector	0.5µl	
T4 DNA Ligase	1.0µl	(1 Weiss unit)
10X T4 DNA Ligase Buffer	1.0µl	

Volume made up to 10µl by Nuclease Free Water (NFW). The ligation was performed at 4°C for 16 h.

b. Preparation of *E. coli* Top10 Competent Cells

- i. Two LB agar plates were prepared, one with ampicillin and another without ampicillin.
- ii. The *E. coli* Top10 cells, from frozen stock (grown in M9 media), were streaked on both the plates and incubated overnight at 37°C.
- iii. Bacterial colonies which appeared on LB agar (without ampicillin) but not on LB agar with ampicillin were picked and grown into 5 ml of LB broth without ampicillin. The culture was then incubated overnight at 200 rpm at 37°C in a shaker-cum- incubator.
- iv. 2.0 ml (1/100th volume) of the overnight-grown culture was inoculated in 200 ml of LB broth and further incubated at 220 rpm until the culture reached an O.D. of 0.3-0.4 at A₆₀₀ nm (usually for 3-4 h).

- v. The culture was chilled on ice and the cells were harvested by centrifuging at 6000 xg for 15 min at 4°C.
- vi. The cell pellet was resuspended in 20 ml (1/10th volume) ice-cold solution of sterile 100mM CaCl₂ and was kept on ice for 30 min.
- vii. The cells were again pelleted by centrifuging at 6000 xg for 15 min at 4°C.
- viii. Finally, the pellet was resuspended in 1.75 ml of ice-cold 10mM CaCl₂ solution with 0.75 ml of sterile glycerol.
- ix. The suspension was gently mixed, aliquoted @ 100-200µl/tube in 1.5ml microcentrifuge tubes and stored at -80°C for further use.

c. Transformation of Competent Cells

- i. The stored competent cells were thawed on ice (usually for 5-10 min).
- ii. 5µl of ligated product was added to it, the tube was flicked gently for homogenous mixing and was kept on ice for 20 min.
- iii. The cells were then heat-shocked at 42°C for 45-50 sec in a waterbath and then immediately transferred on ice and kept for 2 min.
- iv. 850µl of SOC (Appendix) medium was added to it and incubated at 37°C for 90 min at 200 rpm in a shaker-cum-incubator.
- v. 200µl of the transformant culture (from step iv) was spread over the freshly prepared LB/Ampicillin/X-gal/IPTG (Appendix) plates, which were then incubated for 16-18 h at 37°C.

d. Screening of the Recombinant Clones

pGEM-T Easy vector allows easy screening of the recombinant clones among the transformants. Recombinant clones represented as white colonies were picked up, inoculated into 5 ml of LB broth containing ampicillin (100µg/µl) and grown overnight at 37°C in a shaker incubator for plasmid isolation.

3.2.8. Isolation of plasmid

- i. Five ml of overnight culture (from previous step) was taken in a 2.0 ml microcentrifuge tube and centrifuged at 8,000 xg for 2 min.
- ii. Supernatant was discarded and the pellet was resuspended with 250µl of MX1 by vortexing.
- iii. 250µl of MX2 was added to it and was mixed gently by inverting until lysate becomes clear. The mixture was kept for 1- 5 min at room temperature (RT).
- iv. 350µl of MX3 was added to it, inverted upside down to mix gently. The mix was then centrifuged at 13000 xg for 5 min and the supernatant was transferred to a SPIN^m Column onto a collection tube.
- v. The spin column was centrifuged for 60 sec at 8000 xg. The flow-through was discarded; column was washed once with 0.5 ml WF Buffer by centrifuging for 60 sec. The flow-through was discarded.
- vi. The column was washed once with 0.7 ml WS Buffer by centrifuging for 60 sec and the flow-through was discarded.
- vii. The column was centrifuged at full speed for another 3 min to remove residual ethanol.
- viii. The column was placed onto a fresh 1.5 ml microcentrifuge tube, 50µl of NFW was added onto the column which was then left to stand for 1-2 min and centrifuged for 1-2 min to elute DNA.
- ix. The eluted DNA was preserved at -20°C for future use.

3.2.9. Characterization of putative recombinant plasmid

Isolated putative recombinant plasmids were further characterized by digestion with appropriate restriction enzymes and sequencing to check the presence of desired insert.

(a) *Restriction enzyme analysis of cloned plasmid*

Recombinant pGEM-T easy vector was digested with *EcoR* I enzyme for insert confirmation. The reaction mixture contained the following reagents was used in the RE analysis.

10X Buffer	1.5µl
<i>EcoRI</i>	1.0 µl (10 IU)
Isolated Plasmid DNA	12.5µl
Total volume	15µl

The reaction mix was prepared in a 0.5ml microcentrifuge tube. The contents were mixed thoroughly followed by a brief spin. The tube was then incubated overnight at 37°C. The RE digested product was then run in a 1.0% agarose gel with molecular weight DNA marker to check the release of the insert of desired size from recombinant plasmid DNA. The clones, releasing the exact size of insert, were selected as positive clones for further sequencing purpose.

850µl of the positive bacterial culture was mixed with 150µl of sterile glycerol and stored at -80°C, while extracted plasmid DNAs was stored at -20°C until further use.

(b) Sequencing of the cloned target

The transformants containing putative recombinant vector plasmid were sent to Delhi University South Campus as stab culture for sequencing where it was done commercially using automated sequencer ABIPRISM®3100 (AME Bioscience, Toroed, Norway).

3.2.10. Sequence analysis of amplicons

The sequences were initially analyzed using NCBI online BLAST Server (Altschul *et al.*, 1997) to identify the sequence identity. Based on the BLAST results of BLAST server, sequences were further compared with nucleotide sequences of other members of *Orthopoxvirus* available in the GenBank database. Sequence identities among all the orthopoxvirus were determined using the program MegAlign of the Lasergene 6.0 software (DNASTAR Inc., Madison, USA).

3.3. TaqMan probe based QPCR

The TaqMan probe based QPCR reaction was carried out for target gene (C18L) in 50µl of reaction mix. A typical PCR reaction mix contains the following reagents (Invitrogen, Carlsbad, California, USA) and was performed in a thin-walled real-time PCR tubes (Axygen, USA).

Component (stock)	Amount (volume)
10X PCR Buffer	5.0 µl
50mM MgCl ₂	2.0µl
10mM dNTPs	1.0 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10 pmol)	1.0 µl
Template DNA	2.0 µl
<i>Taq</i> DNA Polymerase (5U/µl)	0.2 µl
Probe (10pmol)	1.0 µl
Nuclease free water	36.8 µl
Total reaction volume	50.0 µl

The contents were mixed thoroughly and spin briefly. The tubes were placed in QPCR cabin. Different amplification program were used with buffalopox-specific primer set (Table 3).

Probe is HEX labelled (reporter dye). Excitation maximum and emission maximum are 535 nm 555 nm wavelengths respectively. In QPCR the reading was taken at 555nm at end point.

BHQ-1 (quencher dye): Absorbance is maximum at 534 nm wavelength.

Table 3: Thermal conditions used for real time PCR

Primers used	Thermal profile				Expected size of amplicon (bp)
	1	2	3	4	
C18L-F/ C18L-R	95°C	95 °C	61 °C	40 cycles	138
TaqMan Probe	4 min	30 sec	1 min	(step 2 to 3)	

3.3.1. Virus titration

Virus titration was carried out in 96 well microtiter plates using Vero cells before and after freeze drying of vaccine. Briefly,

- Vero cells were subcultured and 100µl of the cell suspension was added to each well (10⁶ cells/ml)
- The plates were incubated at 37°C in the presence of 5% CO₂
- The virus was diluted 10-fold in EMEM with 2% FBS
- 100µl of virus suspension was added to each well keeping five replicates/dilution
- Four wells were kept as control per plate
- The plates were incubated at 37°C in the presence of 5% CO₂
- Changed the media on every alternate days with EMEM containing 2%FBS
- Reading was taken on 2nd day of titration
- End points were calculated (Reed and Muench, 1938)

3.3.2 Freeze-drying of vaccines

a. *Preparation of stabilizer*

Three different stabilizers were used for the preparation of vaccines. The stabilizers were prepared as per standard protocols for freeze-drying of the vaccine. Step-by-step protocols are briefly described below.

Stabilizer I (LS stabilizer)

LS (Lactalbumin hydrolysate +sucrose) stabilizer was prepared as described by Mariner *et al.* (1990).

- Twenty gram of lactalbumin hydrolysate (LAH, M/s Difco, USA) was taken in a flask.
- 150ml of HBSS (Appendix) was added to it and stirred on a magnetic stirrer for 10 minutes.
- Then the flask was kept in a waterbath at 37°C until LAH was dissolved completely.
- After cooling the solution to room temperature, 40 gm of sucrose (M/s SRL Pvt Ltd, India) was added and stirred on a magnetic stirrer.
- Finally the volume was made up to 200 ml with HBSS and pH was adjusted at 7.2 with 0.1N NaOH.
- The solution was filtered using 0.45µm Seitz filter and stored at 4°C until use.

Stabilizer II (LHT stabilizer)

LHT stabilizer was prepared using the same method as LS stabilizer except 40g of trehalose was added instead of sucrose in LS stabilizer

Stabilizer III (TAA stabilizer)

Stabilizer III (TAA) was prepared according to Adebayo *et al.* (1998) with modifications.

Solution I

Trehalose dihydrate	4.688g
L- Alanine	0.0267g
L-Histidine	0.0466g

The contents were dissolved in 8 ml of 1X PBS and sterilized using 0.45µm Seitz filter and stored at 4°C until use.

Solution II

CaCl ₂ 2H ₂ O	0.05g
MgSO ₄ 7H ₂ O	0.0038g

The contents were dissolved in 50 ml of 1X PBS and sterilized using 0.45µm Seitz filter and stored at 4°C till use.

- ✓ For preparation of the stabilizer II, 8 ml of solution I was added to 2ml of solution II and pH was adjusted to 7.4 using IM Tris-HCl.
- ✓ For preparation of stabilized vaccines, 9.4 ml of stabilizer III mixed with 10 ml of freeze-thawed virus suspension.

b. Lyophilization

Lyophilization was carried out using Edwards Modulyo 4K freeze drier. Vaccine and stabilizer were mixed in equal volume. Taking sterile precautions. 1.0 ml of vaccine with stabilizer was dispensed into sterile freeze-drying vials using an automatic dispenser. The vials were half-closed with sterile rubber stopper to provide a vent during drying of the vaccine. The half-

closed vials were then kept at -80°C for pre-freezing overnight. Freeze- drying was carried out as per the following procedure.

- Freeze drier was switched on 15 minutes prior loading the vaccine to bring the condenser temperature at -60°C .
- At the same time, the vacuum pump was also run to heat the oil to reach the operating temperature.
- After 15 minutes pre-frozen vaccine vials were loaded into lyophilizer trays.
- The pump was stopped and the vaccine vial filled trays were placed in the freeze-drier.
- The vacuum pump was switched on to raise the vacuum in the shelf.
- The vacuum reached a safe limit of $10^{-0.06}$ torr in 1 hr and maintained throughout the lyophilization period with little fluctuation.
- After 48 hrs, the stopper of the vaccine vials were closed at maximum vacuum and the vials were sealed manually with aluminium cap.
- Sealed vials were tested by vacuum torch for vacuum and kept at -20°C till further use. Each vial consisted of 100 doses of vaccine.

3.3.5 Sterility test of stabilizers and vaccine

Sterility testing of stabilizers and vaccine was done as per OIE (OIE 2008). Soya casein digest media (Appendix) was used for the purpose. Test tubes containing 5 ml of Soya casein digest media was used. Stabilizer/ vaccine in 0.5 ml volume was added to four tubes, out of which two tubes each were maintained at 25°C and 37°C for detection of fungal and bacterial contamination, if any, respectively. Two tubes without stabilizer/ vaccine were kept for each temperature as controls. The tubes were incubated for 7 days for the growth of bacteria and fungi, if any.

3.3.6 Measurement of residual moisture

Residual moisture (RM) in the freeze-dried product was measured by thermo gravimetric method (Worral *et al.*, 2001). In brief,

- ✓ Freeze-dried vaccine vials were weighed in an electronic balance.
- ✓ Rubber stopper of the vials were opened and weighed.
- ✓ Vials were dried completely in hot air oven at 80°C for 20 hrs.
- ✓ Vials with dried vaccine were weighed and then washed thoroughly.
- ✓ Empty vials were kept for drying again at 80°C for 30 minutes and weighed again.

Percentage of residual moisture was calculated using the following formula:

Initial weight of vial with freeze dried product + rubber stopper	A
Weight of aluminium seal + rubber stopper	B
Weight of vials after drying for 20hrs at 80°C	C
Weight of empty vials	D

3.5 Thermo-stability testing of buffalopox vaccine

The stability of buffalopox vaccine with different stabilizers in the freeze-dried form as well as after reconstitution was studied at various temperatures.

3.5.1 Stability of freeze-dried vaccine

Ample number of freeze-dried vials of vaccine with each stabilizer was exposed at 25°C, 37°C in incubators and 45°C in a dry oven. The vaccine vials were sampled from incubator (25°C) on months 1, 2 and 3; from 37°C on days 3, 5, 7, 10 and 14; and from 45°C at 6 hr interval up to 48 hr. Exposed samples were reconstituted with 1 ml of serum free EMEM and titrated in Vero cells. For each exposure, three samples were titrated and their average log titer was calculated (Reed and Muench, 1938).

3.5.2 Stability of reconstituted vaccine

Freeze-dried vials of each stabilizer were reconstituted with three diluents viz. phosphate buffer saline (PBS), 1M MgSO₄ and normal saline solution (NSS). For each stabilizer, three vials were taken and reconstituted separately with 1ml each diluent. Reconstituted vaccines were then exposed to 4°C (ice), 25°C, 37°C and 45°C. Samples from each temperature were taken out at 6 hourly intervals up to 48 hours and titrated immediately.

3.6 Potency estimation of live attenuated buffalopox vaccine by QPCR

3.6.1 Establishment of standard reference sample

BPXV (Vij/96, P-49) batch of live attenuated buffalopox vaccine produced at poxvirus disease laboratory; Division of Virology was taken as for reference purpose. The reference vaccine was assigned with the TCID₅₀ by virus titration in confluent Vero cell monolayer seeded in 96 well plate and vaccine virus was stored at – 20°C until further use. The titer of virus was rechecked periodically to know storage effects on the vaccine virus titre. The procedure used is as follows.

- i. The tenfold dilution of reference vaccine virus was carried out in Eagle's minimum Essential medium.
- ii. 100 µl of each dilution was added per well in five replicates.
- iii. One positive control with virus and a negative control with no virus were also maintained.
- iv. Media was changed with the EMEM containing 1% BCS twice, at 48 hour post infection and 96-hour post infection.
- v. The appearance of CPE in all the wells was recorded on 6-8 days post infection
- vi. At the end of incubation, the medium was removed and cells were fixed with 10 % formalin for 1 hour and each well is overlaid with 100 µl of 1% crystal violet stain and allowed for 15 min.
- vii. Plate was rinsed with distilled water to remove the stain completely and air dried
- viii. Based on the extent of CPE the titer of virus was determined using Reed and Muench (1938) formula

The above procedure was carried out repeatedly for three times and average TCID₅₀ was calculated to assign the titer of virus for the reference vaccine batch.

3.6.2 Estimation of critical time for harvest

Critical time(ct) to harvest the infected Vero cells to estimate the titer of test vaccine batch by QPCR was established by infecting the different dilution buffalopox virus to confluent

Vero cell monolayer followed by harvesting at different hour interval and carrying out QPCR upon extracting the DNA from each dilution. The detailed procedure is as follows.

- i. Buffalopox (P-49) vaccine virus was diluted ten-fold in EMEM without serum.
- ii. 100 µl of each virus dilution ranging from neat to 10^{-4} were added each well of six well plate having a confluent Vero cell monolayer. One of the well was kept as Vero cell control. Plates were incubated at 37°C for 1hour.
- iii. Plates were removed from incubation. Inoculum was discarded using separate tips followed by two-time wash with EMEM. Finally 2 ml of EMEM maintenance medium was overplayed and plates were transferred to CO₂ incubator maintained at 37°C.
- iv. Each plate was harvested by freeze thawing at 12h, 24h, 36h, 42h, 46h, 60h, 72h, 84h, 96h, and 120h and DNA was isolated.
- v. The QPCR was carried out with the DNA isolated from each hour interval to establish the standard curve.
- vi. The standard curve of particular hour interval having the maximum slope was considered as the critical time for harvest.

The above procedure was repeated thrice and average slope was calculated. The standard curve of particular hour interval having maximum slope was considered as the critical time for harvest.

3.6.3 Estimation of infectious viral load in vaccine batches

Infectious viral load of test vaccine batches were established against the reference vaccine batch by QPCR. The complete protocol is as follows.

- i. The reference and test vaccine batches (six different batches of buffalopox vaccine) were diluted serially tenfold in EMEM.
- ii. The diluted reference as well as test vaccine virus were inoculated into two separate six well plates.
- iii. Both the plates were harvested at 30-hour post infection and DNA was isolated from all the dilutions.

- iv. QPCR was carried out with DNA from different dilutions of reference as well as test sample to establish the standard curve.
- v. The titer for the test vaccine batch was assigned by comparing with the titer of the standard reference sample in standard curve established in QPCR.
- vi. The same protocol was repeated for different batches of live attenuated buffalo pox vaccine.

3.6.4 Comparision of virus titres obtained by TCID₅₀ and QPCR methods

The vaccine titers obtained by QPCR was compared with the TCID₅₀ titers obtained by titration in Vero cells and analysed statistically Snedecor and Cochran (1994).

3.6.5 Statistical analysis

The degradation values of buffalopox vaccine (both freeze-dried as well as reconstituted) after exposure to various temperatures for different time duration, regression analysis was carried out using the regression module of Microsoft Excel software. Q-PCR results obtained for vaccine batches were compared with that of virus titres obtained by TCID₅₀. For student's t-distribution, the standard table was referred from Bailey (1995).



4.1 Revival and Bulk production of buffalopox vaccine virus

The buffalopox vaccine virus (BPXV Vij/96) at passage levels P49 was revived in Vero cells. The virus produced characteristic cytopathic effects viz., rounding, ballooning, increased refractility and detachment of Vero cells from the surface. Bulk production of the vaccine virus was done in roller flasks having twenty-four hour confluent. Vero cell monolayer was infected with buffalopox vaccine virus (BPXV). Upon appearance of cytopathic effects characteristic of buffalopox virus, the infected Vero cells were harvested by three cycles of freezing and thawing.

4.2 Identity of the vaccine virus

The vaccine virus (BPXV Vij/96) used in the present study was propagated in Vero cells and tested for its identity based on characteristics CPE, PCR (conventional and real time) as well as by cloning and sequencing of truncated ankyrin repeat protein gene(C18L). Virus induced CPE was characterized by rounding, ballooning, increased refractility and detachment of Vero cells. CPE was initiated by 24 hour post infection and progressed to 80% by 36-48 hours (Fig. 1 A, B). Further, it was confirmed as *buffalopox virus* by amplification of *C18L gene* in conventional PCR (Fig 2). As well, amplicons of C18L gene of the vaccine virus was cloned into pGEM-T^{easy} vector and sequenced commercially. The C18L gene nucleotide sequences of the vaccine virus was compared with that of the sequences available in the GenBank database. Further, sequence analysis of C18L genes showed 100% of similarity with other buffalopox viruses at nt level (Table 4, Fig 3 & 4). The aforementioned tests were

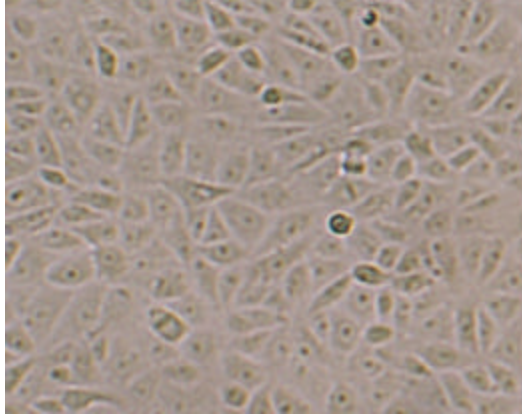


Fig. 1A : Confluent monolayer of Vero cells

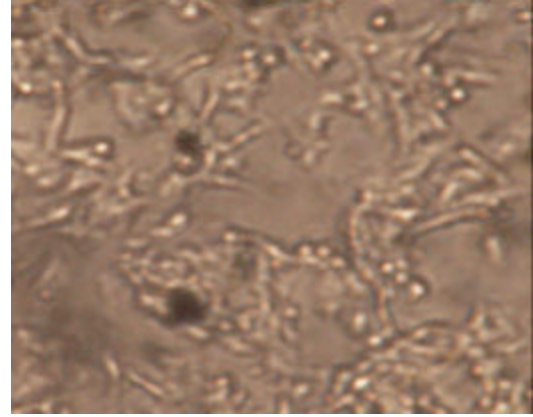


Fig. 1B : Vero cells infected with BPXV (Vij/96) showing characteristics CPE

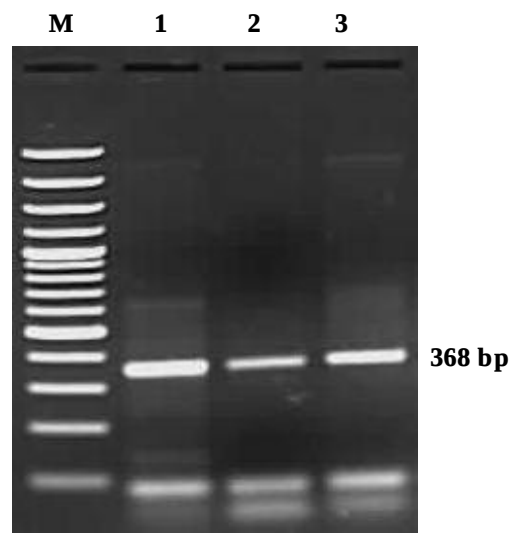


Fig. 2 : Agarose gel electrophoresis of C18L gene PCR of BPXV vaccine virus (BPXV Vij/96)

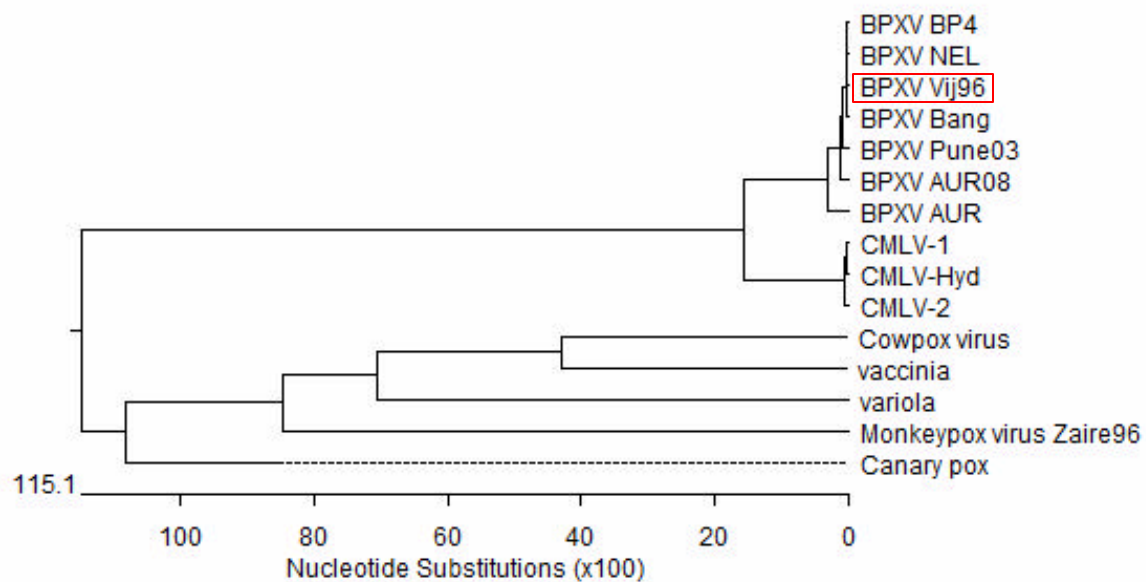


Fig. 3 : Phylogenetic tree constructed based on the C18L gene sequence of BPXV.

		Percent Identity																	
Divergence		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15			
	1			99.7	99.5	99.5	99.7	99.5	59.5	59.8	59.8	6.5	12.0	11.4	12.0	16.2	1	BPXV AUR	
	2	0.3			99.5	99.7	99.7	100.0	99.7	59.8	60.1	60.1	16.3	12.0	11.4	12.0	16.2	2	BPXV AUR08
	3	0.5	0.5			99.7	99.7	99.5	99.7	59.8	60.1	60.1	6.5	12.0	11.4	12.0	21.2	3	BPXV Bang
	4	0.5	0.3	0.3			100.0	99.7	100.0	59.0	79.6	59.3	7.2	10.2	9.7	12.0	16.2	4	BPXV BP4
	5	0.5	0.3	0.3	0.0			99.7	100.0	60.1	60.3	60.3	16.3	12.0	11.4	12.0	16.2	5	BPXV NEL
	6	0.3	0.0	0.5	0.3	0.3			99.7	59.8	60.1	60.1	16.3	12.0	11.4	12.0	16.2	6	BPXV Pune03
	7	0.5	0.3	0.3	0.0	0.0	0.3			60.1	60.3	60.3	16.3	12.0	11.4	12.0	16.2	7	BPXV Vij96
	8	27.7	27.4	28.2	29.1	27.8	27.4	27.8			99.6	99.8	11.3	8.5	9.3	12.0	11.3	8	CMLV-1
	9	26.9	26.5	27.4	28.4	26.9	26.5	26.9	0.4			99.8	11.3	8.7	9.1	12.0	11.3	9	CMLV-2
	10	27.3	26.9	27.8	28.8	27.4	26.9	27.4	0.2	0.2			11.3	8.7	9.1	12.0	11.3	10	CMLV-Hyd
	11	133.9	135.5	137.6	144.0	137.6	135.5	137.6	139.8	138.2	139.8			3.5	9.4	16.7	27.5	11	Canary pox
	12	211.9	216.8	225.9	203.4	225.9	216.8	225.9	234.7	234.7	234.7	132.3			1.9	99.3	46.8	12	Cowpox virus
	13	190.9	189.3	194.4	205.1	194.4	189.3	194.4	172.8	172.8	175.3	130.9	187.7			30.0	11.7	13	Monkeypox virus Zaire96
	14	293.3	350.0	350.0	350.0	350.0	350.0	350.0	350.0	350.0	350.0	100.8	0.7	133.3			14.7	14	vaccinia
	15	250.5	272.0	350.0	350.0	350.0	272.0	350.0	163.2	163.2	163.2	107.9	103.3	135.5	93.5			15	variola
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15				

Fig. 4 : Sequence identity of BPXV based on C18L gene sequence with othe related viruses.

found enough to confirm the identity of the vaccine virus as buffalopox virus and was used further.

Table 4: NCBI BLAST result of BUFFALO POX virus ankyrin repeat protein C18L gene

Accession	Description	Query coverage	Maximum identity
EF205284	Buffalo pox virus isolate BPXV-VIJ96 nonfunctional C18L-like protein gene, partial sequence	100%	100%
EF205286	Buffalo pox virus isolate BPXV-NEL nonfunctional C18L-like protein gene, partial sequence	100%	100%
EF205289	Buffalo pox virus isolate BPPXV-BP4 nonfunctional C18L-like protein gene, partial sequence	100%	100%
FJ748498	Buffalopox virus C18L gene, partial sequence	100%	99%
EF205288	Buffalopox virus isolate BPXV-BANG nonfunctional C18L-like protein gene, partial sequence	100%	99%
EF205285	Buffalopox virus isolate BPXV-PUNE03 nonfunctional C18L-like protein gene, partial sequence	100%	99%
EF205287	Buffalopox virus isolate BPXV-AUR nonfunctional C18L-like protein gene, partial sequence	100%	99%
EU410304	Vaccinia virus GLV-1h68, complete genome	100%	97%
AY678275	Vaccinia virus strain LC16m8, complete genome	100%	100%
AY678277	Vaccinia virus strain LC16mO, complete genome	100%	100%
AY678276	Vaccinia virus strain Lister, complete genome	100%	100%
AM501482	Vaccinia virus Ankara strain chorioallantois vaccinia virus Ankara (CVA), complete coding genome	61%	96%
M35027	Vaccinia virus Copenhagen, complete genome	100%	95%

4.3 Thermo-stability evaluation

4.3.1 Production of vaccine

Adsorption method was followed for production of vaccine. Upon infection on confluent monolayer of Vero cells, CPE was initiated after 24 hour post infection (dpi) and more than 80% CPE appeared on 2 dpi. In order to prepare the freeze-dried vaccine with various stabilizers, viz LS, LHT and TAA three different batches of vaccines were produced. Batch I

was used for preparing the freeze-dried buffalopox vaccine containing LS stabilizer, whereas, Batch II was used for preparing the freeze-dried buffalopox vaccine containing stabilizer LHT while batch III was prepared using TAA stabilizer. The virus vaccines were titrated after two cycles of freezing-thawing and also immediately after freeze-drying. The titers of each batch before and after freeze-drying and other criteria which attribute to the quality of vaccine are depicted in Table I. It is clear from the table that the initial harvest titers of all batches of vaccines were $>10^{6.0}$ TCID₅₀/ml. The loss of virus during freeze-drying conditions for all the stabilizers was within the acceptable limits. The vaccine comprising stabilizer TAA was found to have the least residual moisture (RM) whereas, vaccine containing LS stabilizer found to have highest RM (Table. 5).

4.4. Thermo-stability of freeze-dried buffalopox vaccines

Sufficient vials of lyophilized vaccine were exposed at different temperature over different periods.

4.4.1 Stability at 4°C

Virus titration was carried out to determine the infectivity of the freeze-dried vaccines after exposing them at 4 °C for 4 months and sampling at 30 days interval. The infectivity titers thus obtained for each sample of the candidate vaccine viruses are given in Table 6. Regression analysis was done based on these values and the degradation curves are depicted in Fig.5. At 4°C all the stabilizers were found to maintain the required infectivity for the entire period of study.

4.4.1. Stability at 25° C

Virus titration was carried out to determine the infectivity of the freeze-dried vaccines after exposing them at 25° C for 4 months and sampling at 15 days interval. The infectivity titers thus obtained for each sample of the candidate vaccine virus are given in Table 7. Regression analysis was done based on these values and the degradation curves are depicted in Fig. 6.

4.4.2. Stability at 37° C

Samples taken from 37° C at different time intervals were studied for infectivity titer for both the candidate vaccine viruses are tabulated in Table 7. The titers were subjected to regression analysis and the degradation curves are depicted in Fig.7-8. At this temperature,

Table 5 : Comparative quality of freeze dried buffalopox vaccine with various stabilizers

Stabilizer	Before lyophilization	After lyophilization	Loss of Titre	Residual moisture (%)	Appearance
LS	6.85 TCID ₅₀ /ml	6.45	0.4	3.2	Pellet form
LHT	6.85 TCID ₅₀ /ml	6.45	0.4	3.7	Pellet form
TAA	6.85 TCID ₅₀ /ml	6.35	0.5	5.2	Pellet form

LS= Lactalbumen hydrolysate + Sucrose

LHT= Lactalbumen hydrolysate + Trehalose dihydrate

TAA= Trehalose dehydrate + Amino acids + divalent cations

Table 6: Comparison of degradation values of buffalopox vaccine containing various stabilizers at 4°C

		Titre (log ₁₀ TCID ₅₀)		
		LS	LHT	TAA
months post exposure	0	6.45	6.45	6.35
	1	6.45	6.3	6.3
	2	6.2	6.4	6.1
	3	6.4	6.4	6.3
	4	6.3	6.2	6.1
Shelf life ¹		40.85	35.75	26.6
Half life ²		8.5	7.5	6

¹Time required for reaching 5log₁₀TCID₅₀/ml in a 100 dose vaccine preparation, calculated from the regression equation.

²Time required for loss of half of the original titre i.e., 0.3 log₁₀ TCID₅₀ /ml based on degradation constant.

Table 7: Comparison of degradation values of buffalopox vaccine containing various stabilizers at 25°C

		Titre ($\log_{10}$ TCID ₅₀)		
		LS	LHT	TAA
Days post exposure	0	6.45	6.45	6.35
	15	6.4	6.45	6.35
	30	6.45	6.45	6.3
	45	6.25	6.25	5.86
	60	6.05	6.38	5.7
	75	6.45	5.96	6.22
	90	6.65	5.5	5.15
	105	5.75	5.74	5.45
	120	5.3	5.25	4.8
Shelf life ¹		241.07	163.9	126.7
Half life ²		45.45	29.70	25

Table 8: Comparison of degradation values of buffalopoxvaccine containing various stabilizers at 37°C

		Titre ($\log_{10}$ TCID ₅₀)		
		LS	LHT	TAA
Days post exposure	0	6.45	6.45	6.35
	3	6.45	6.45	6.3
	5	6.35	6.3	6.25
	7	6.3	6	5.75
	10	5.85	5.75	5.35
	14	5.4	5.25	4.9
Shelf life ¹		25.67	31.16	14.7
Half life ²		4.6	6.1	2.9

Fig. 5 : BPXV undiluted graphs at 4°C

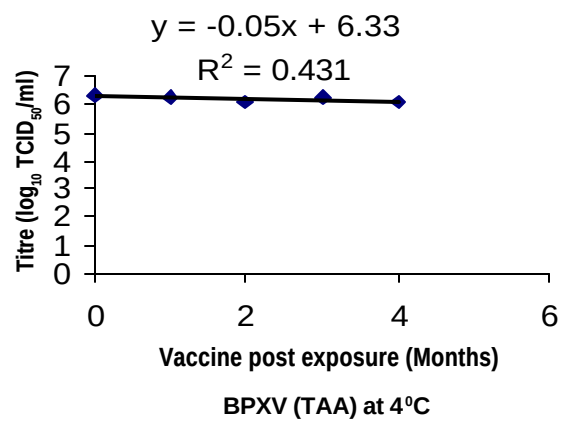
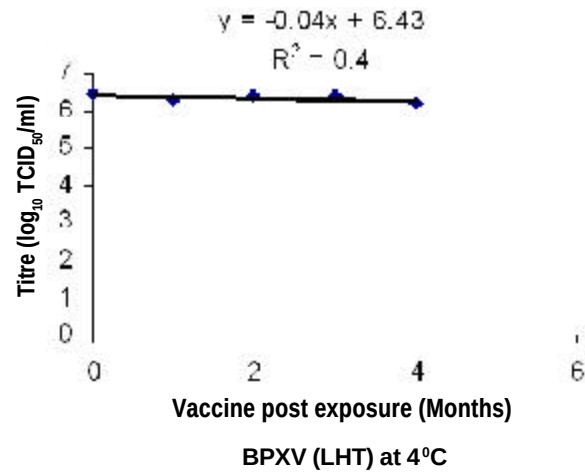
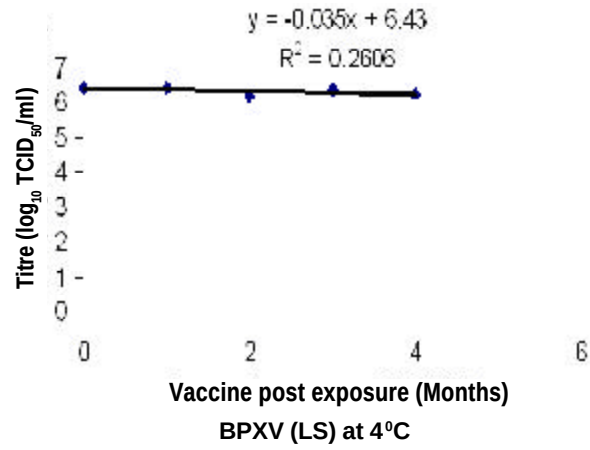
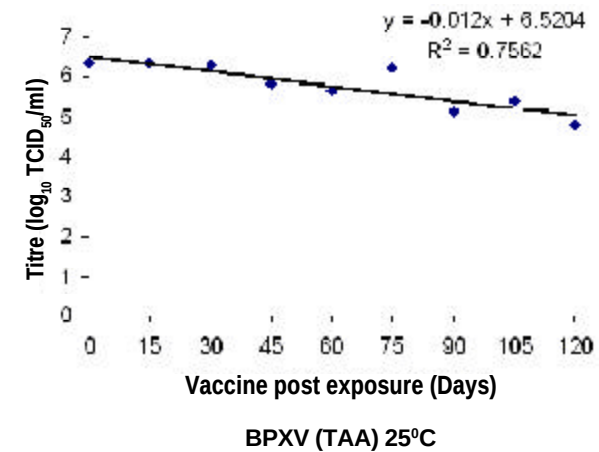
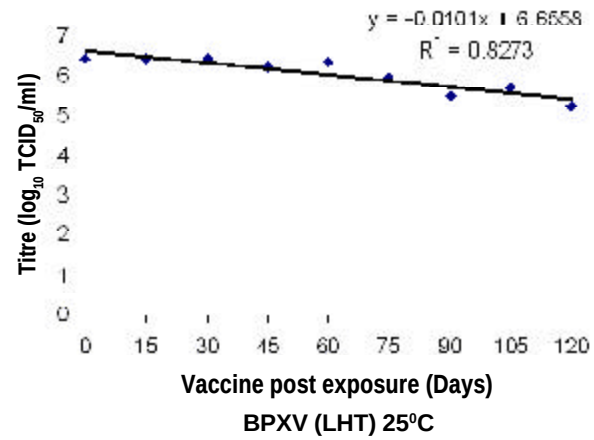
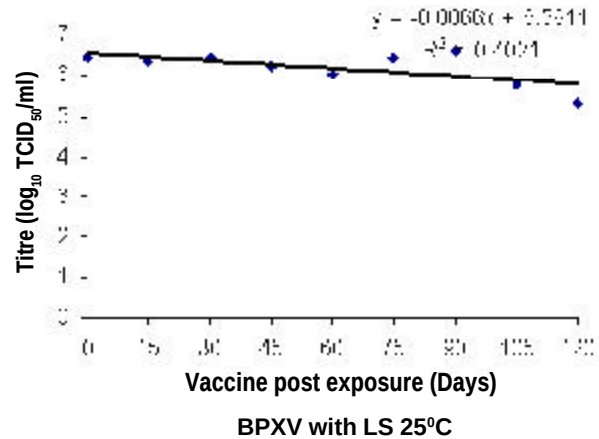


Fig. 6 : BPXV undiluted graphs at 25°C



vaccines with LHT stabilizer showed better stability in terms of its shelf-life and half-life followed by LS and TAA stabilizer.

4.4.3. Stability at 45 ° C

Freeze-dried vaccine vials were exposed at 45 ° C for a period of two days. Sampling were done at a day interval and titrated. Infective titers obtained for both vaccine viruses are shown in Table 9. The titer values were subjected to regression analysis and the degradation curves are depicted in Fig. 8. Vaccines with LS and TAA stabilizer showed comparable results in terms of its shelf-life; however LS stabilizer had a marginal superiority over TAA in terms of its half-life. Vaccines with LHT stabilizer was proved to be a poor stabilizer in comparison to other two, stabilizer.

4.5. Thermo-stability of vaccines after reconstitution

Freeze-dried vaccines stabilized with different stabilizers were reconstituted in 1 ml of each of the four diluents to maintain a shelf-life of 100 doses ($10^5 \text{TCID}_{50}/\text{ml}$)/vial and exposed at 4 ° C, 25 ° C, 37 ° C and 45 ° C for 48 hrs. Infectivity titers for each sample in different stabilizers are shown in Tables 10-12. The regression analysis of the reconstituted vaccines at various temperatures is represented graphically in Fig. 9-20.

All the three stabilizers could maintain titer well above the recommended levels throughout the sampling period under temperatures studied. At lower temperatures, all the diluents were found to work well with the vaccine as evident by maintaining the titer above $5 \log_{10} \text{TCID}_{50}/\text{ml}$. However, the required infectivity titer found to fall marginally at higher temperatures (37 ° C & 45 ° C) as the time of exposure increases. 1M MgSO_4 could not maintain the required titer with any of the stabilizers at higher temperatures.

4.8. Potency estimation of live attenuated buffalopox vaccine

The real-time PCR assay was employed to estimate the potency of live attenuated buffalopox vaccine. A total of six different vaccine batches were employed for the purpose.

Table 9: Comparison of degradation values of buffalopox vaccine containing various stabilizers at 45°C

		Titre ($\log_{10}$ TCID ₅₀)		
		LS	LHT	TAA
Hours post exposure	0	6.45	6.45	6.35
	12	6	5.85	5.4
	24	5.75	5.55	5.3
	36	5.45	5.05	4.65
	48	4.8	4.5	3.85
Shelf life ¹		44.3	36.2	26.3
Half life ²		9.3	7.6	6.2

Buffalo pox data

Table 10 : Thermo-stability of LS stabilized buffalopox vaccine at various temperatures after reconstitution with different diluents

		Titer ($\log_{10}$ TCID ₅₀)											
		4°C				25°C				37°C			
Undiluted Titer	Loss on dilution	PBS	NSS	MgSO4	DSW	PBS	NSS	MgSO4	DSW	PBS	NSS	MgSO4	DSW
		6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45
	6	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL	NIL
	12	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45	6.45
	18	6.15	6.45	6.45	6.45	6.4	6.2	5.8	6.3	6.35	6.1	5.65	6.15
	24	6.45	6.15	6.25	6.45	6.2	6	5.82	6.45	5.85	5.82	5.5	6.35
	30	6.25	6.4	6.2	6.4	6.35	5.8	5.95	6.15	6.25	5.6	6.15	6.35
	36	6	6.25	6.15	6.4	5.9	6.30	5.65	5.65	5.72	6.3	5.82	5.85
	42	6.25	6.40	6.15	6.25	6.3	6.22	5.8	6.3	5.7	5.7	5.6	5.6
	48	6.30	6.35	6	6.20	6.25	6.35	5.2	6.15	5.83	5.7	4.95	5.72
Degradation Constant		NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

PBS- Phosphate buffer saline, NSS- Normal saline solution, MgSO₄- 1M MgSO₄

Table 11 : Thermo-stability of LHT stabilized buffalopox vaccine at various temperatures after reconstitution with different diluents

Titer (log ₁₀ TCID ₅₀)																					
		4°C					25°C					37°C					45°C				
		PBS	NSS	MgSO4	DSW		PBS	NSS	MgSO4	DSW		PBS	NSS	MgSO4	DSW		PBS	NSS	MgSO4	DSW	
Undiluted Titer	Loss on dilution	6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45	
		NIL	NIL	NIL	NIL		NIL	NIL	NIL	NIL		NIL	NIL	NIL	NIL		NIL	NIL	NIL	NIL	
		6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45		6.45	6.45	6.45	6.45	
		12	6.45	6.45	6.45		6.45	6.4	6.2	6.45		6.35	6.2	6.05	6.3		6.15	6.1	5.7	6.2	
		18	6.3	5.75	6.45	6.35		6.32	6.20	5.7	6.2		6.22	6	5.5	6.05		6	6	6.25	5.9
		24	6.45	6.3	6.2	6.35		6.45	5.9	6.22	6.35		5.82	5.62	5.3	5.72		5.8	5.55	5.65	6.15
	30	5.85	6	5.56	6.28		5.9	6.25	6.22	5.72		6.25	6.35	4.9	6.35		6.15	6.2	4.6	5.75	
	36	6.25	6.2	6.15	6.25		6.4	6.32	5.82	6.4		5.85	5.75	4.7	5.78		6.2	5.85	5.23	5.7	
	42	6.3	6.2	5.65	6.25		6.22	5.85	5.75	6.12		5.28	5.7	4.4	5.65		5.75	5.7	4.9	5.82	
	48	6.25	6.15	5.5	6.16		6	6.25	5.35	6.15		5.5	5.75	4.82	5.52		5.6	5.45	4.76	5.4	
Degradation Constant		NSD	NSD	NSD	NSD		NSD	NSD	NSD	NSD		NSD	NSD	NSD	NSD		NSD	NSD	NSD	NSD	

Fig. 7 : BPXV Undiluted graphs at 37°C

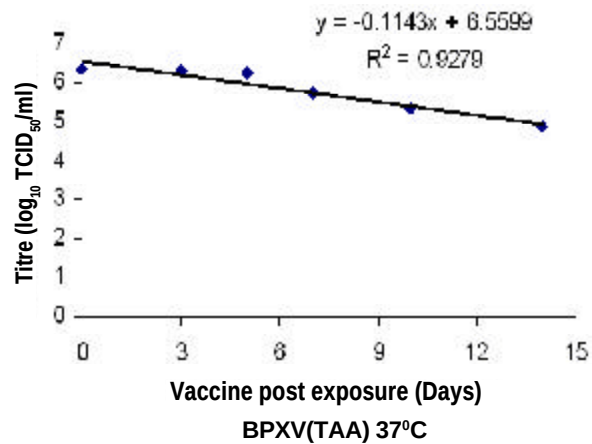
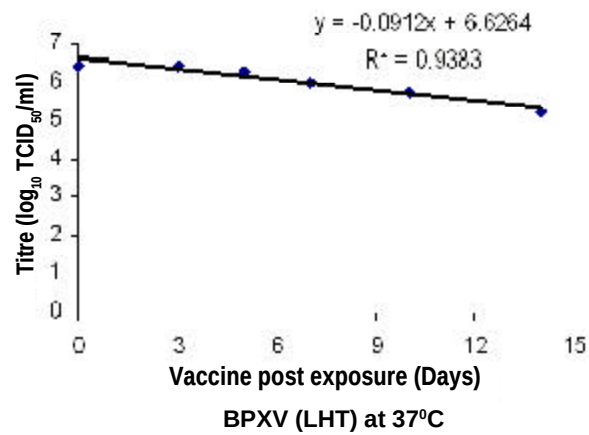
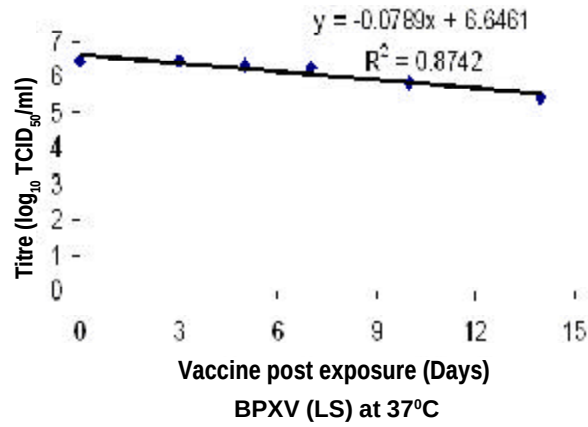


Fig. 8 : BPXV Undiluted graphs at 45°C

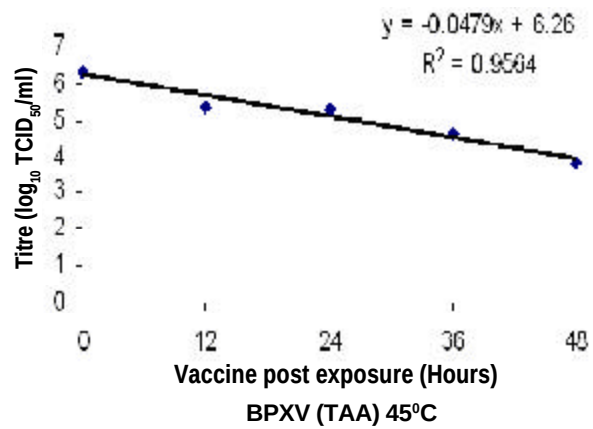
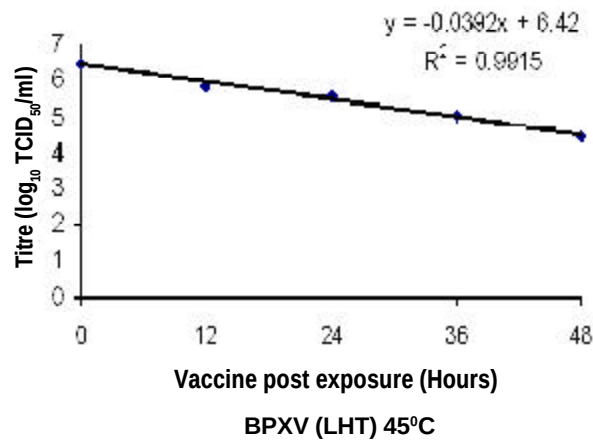
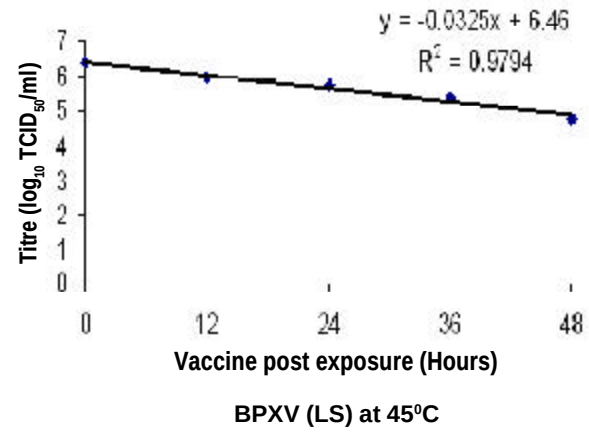
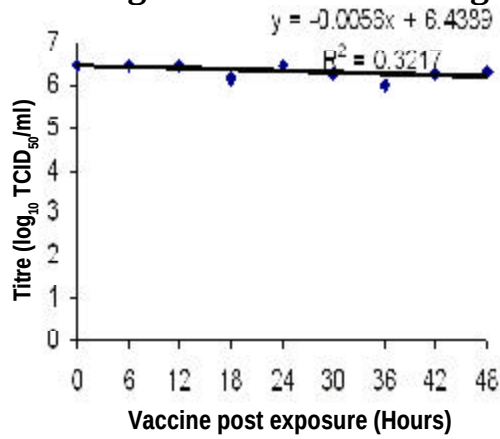
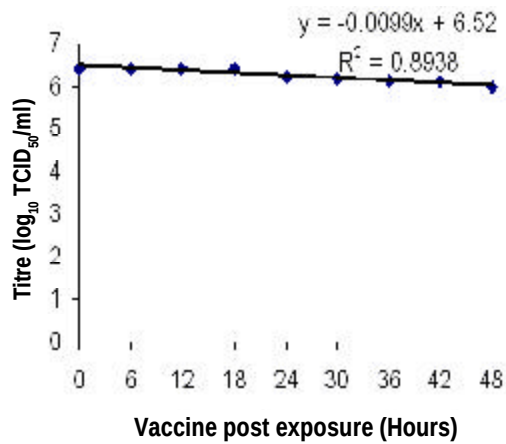
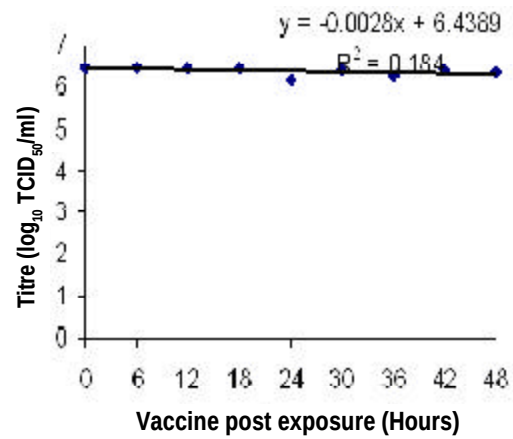


Fig. 9 : BPXV Diluted graphs at 4°C for LS stabilizer



LS + NSS AT 4°C



LS + DSW AT 4°C

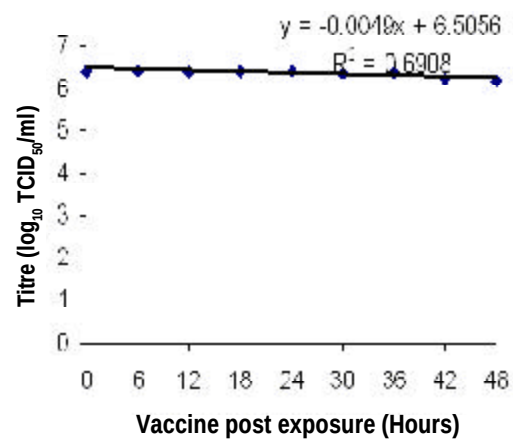
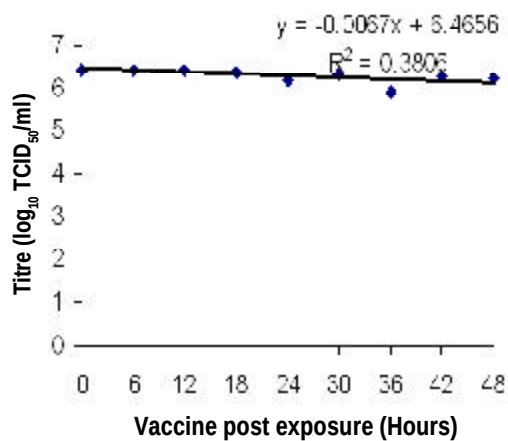
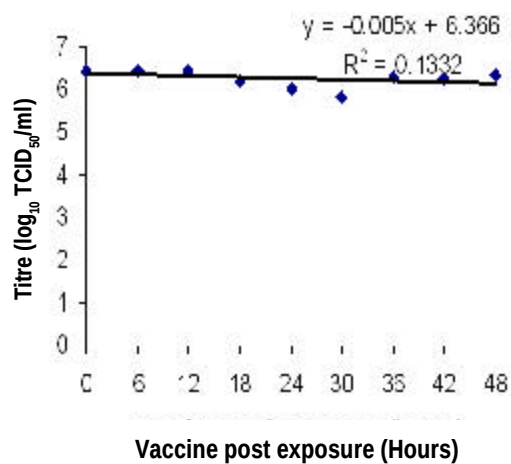


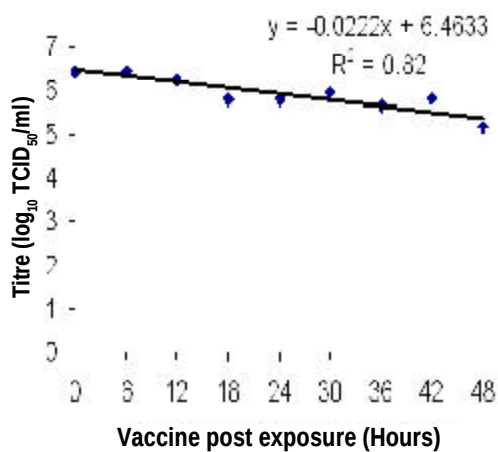
Fig. 10 : BPXV Diluted graphs at 25°C for LS stabilizer



LS + PBS AT 25°C



LS + NSS AT 25°C



LS + MgSO₄ AT 25°C

LS + DSW AT 25°C

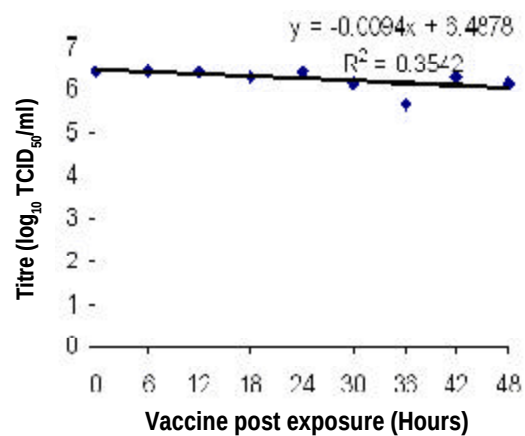
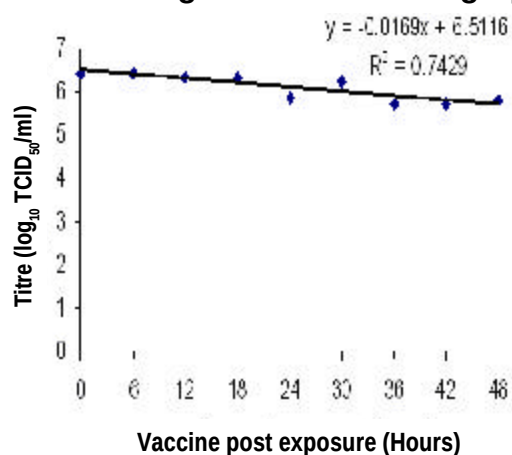
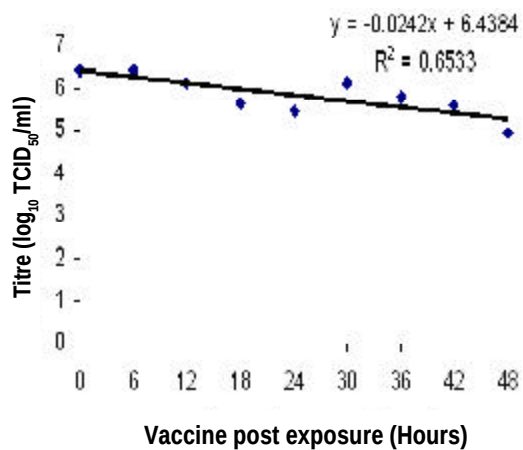
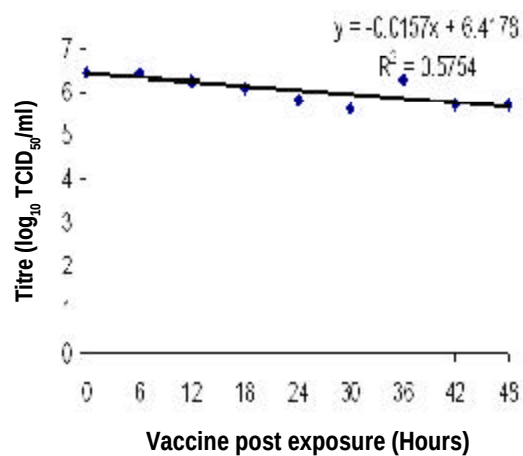


Fig. 11 BPXV Dilutd graphs at 37°C for LS stabilizer



LS + NSS AT 37°C



LS + DSW AT 37°C

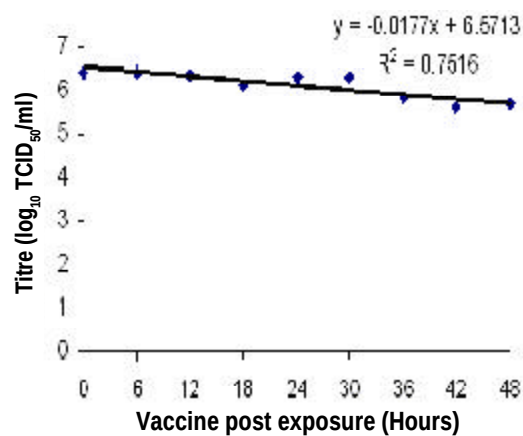
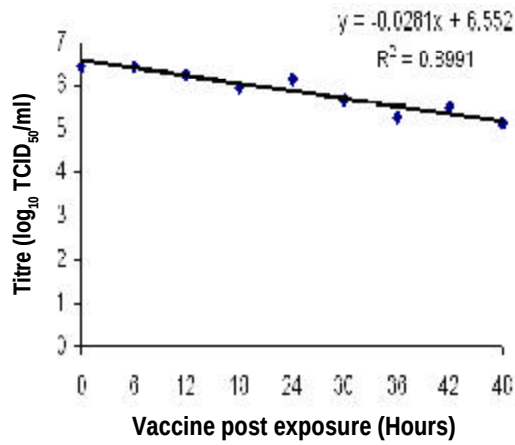
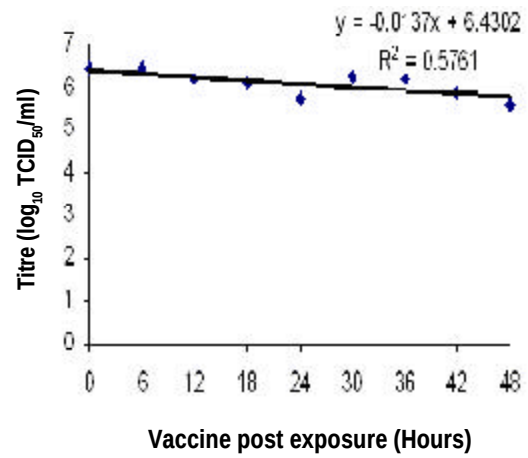


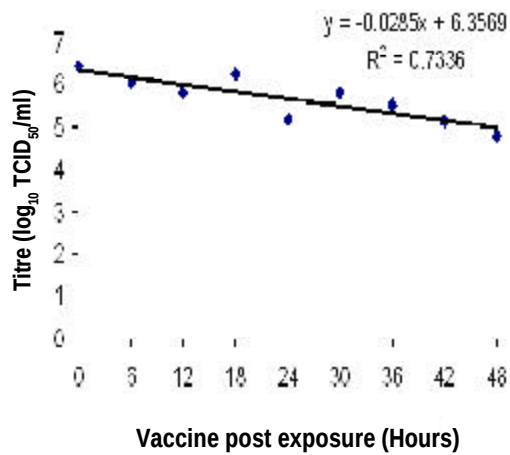
Fig. 12 : BPXV Diluted graphs at 45°C for LS stabilizer



LS + PBS AT 45°C



LS + NSS AT 45°C



LS + MgSO₄ AT 45°C

LS + DSW AT 45°C

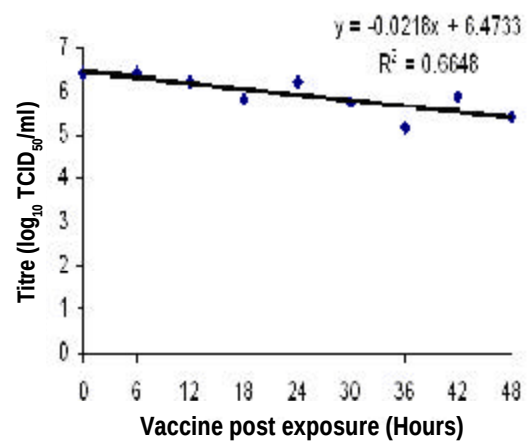
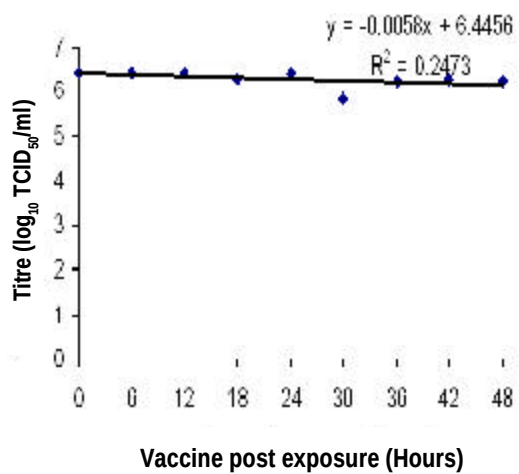
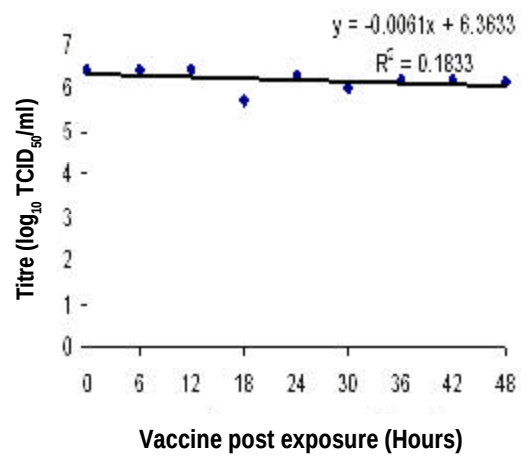


Fig. 13 : BPXV Diluted graphs at 4°C for LHT stabilizer

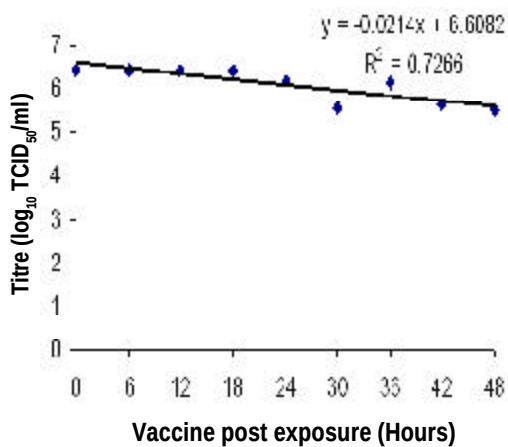


LHT + PBS AT 4°C

LHT + NSS AT 4°C



LHT + MgSO₄ AT 4°C



LHT + DSW AT 4°C

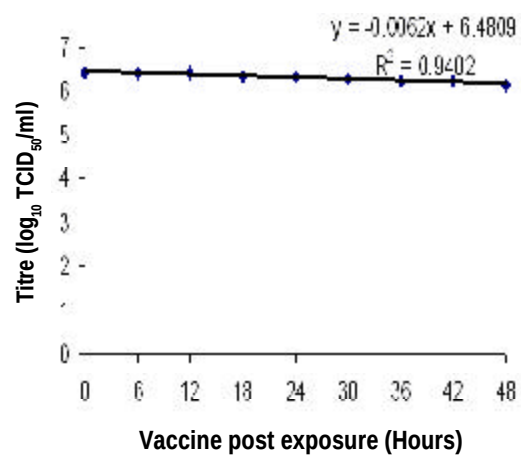
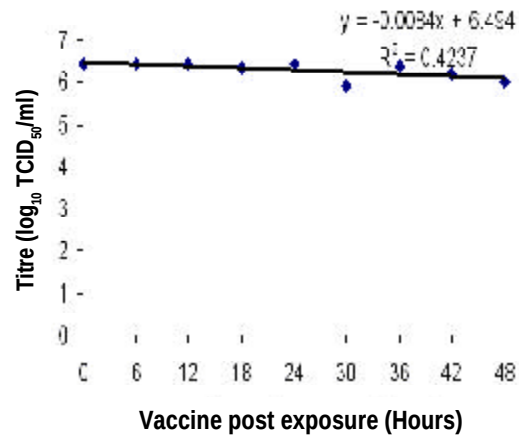
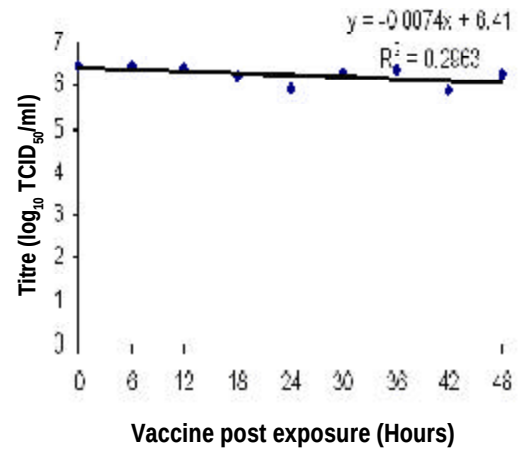


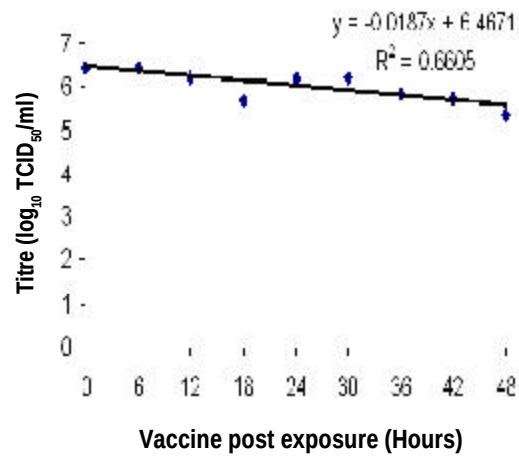
Fig. 14 : BPXV Diluted graphs at 25°C for LHT stabilizer



LHT+PBS 25°C



LHT+NSS 25°C



LHT+MgSO₄, 25°C

LHT+ DSW 25°C

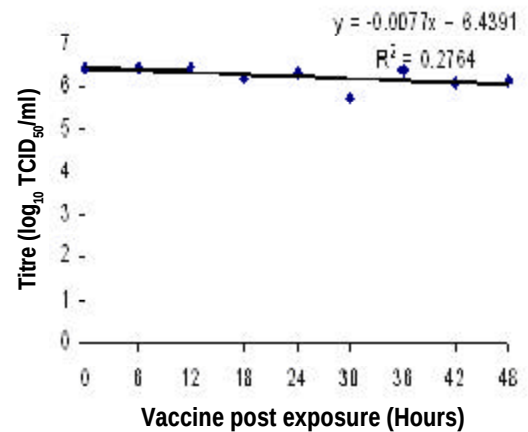
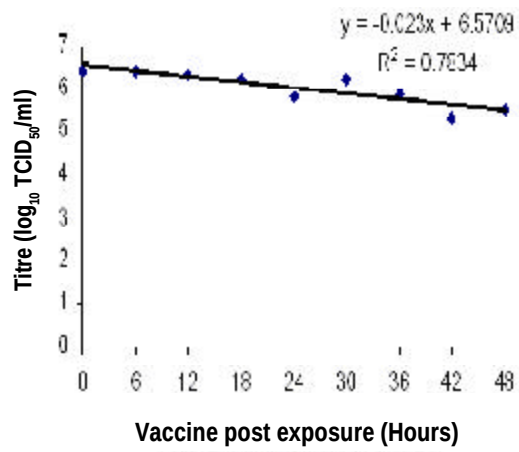
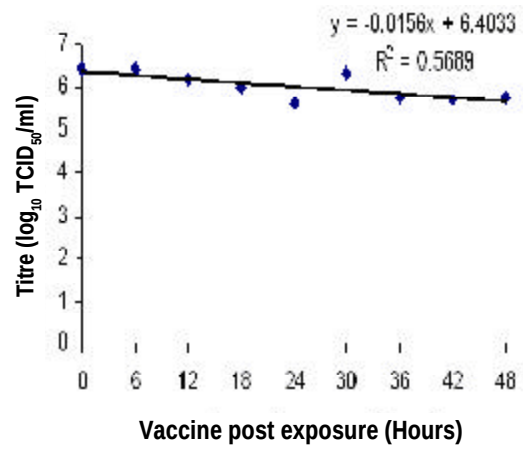


Fig. 15 : BPXV Diluted graphs at 37°C for LHT stabilizer

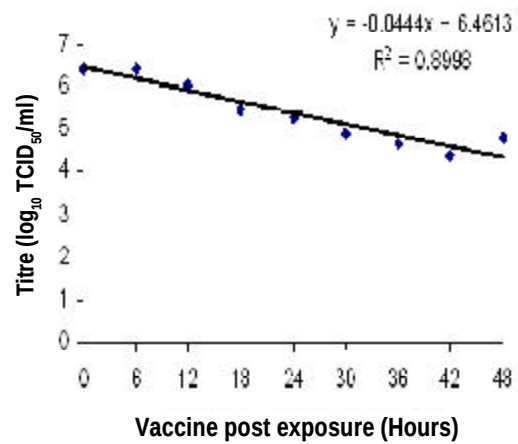


LHT+ PBS 37°C

LHT+NSS 37°C

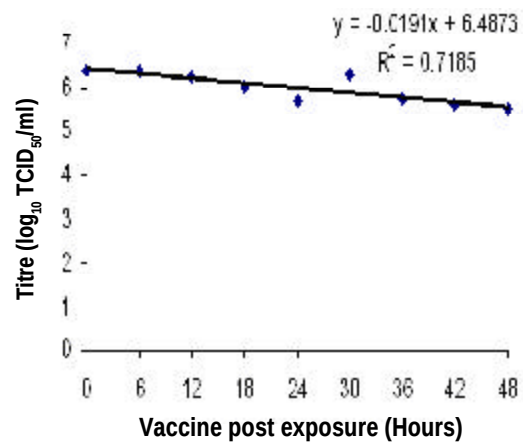


Vaccine post exposure (Hours)



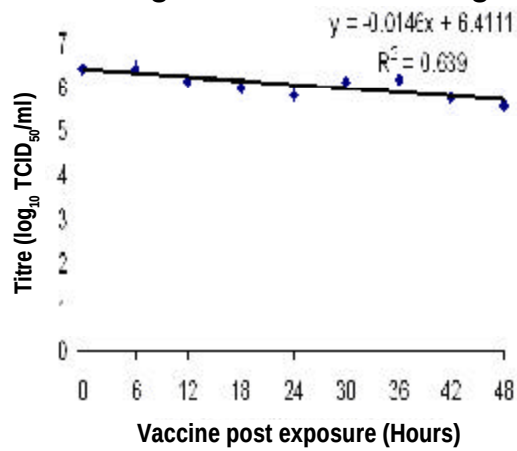
LHT+MGSO4, 37°C

LHT+DSW 37°C

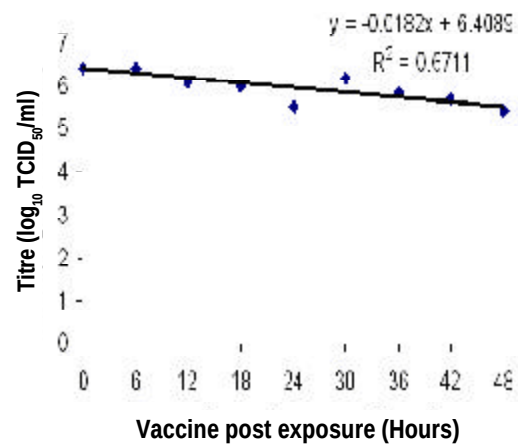


Vaccine post exposure (Hours)

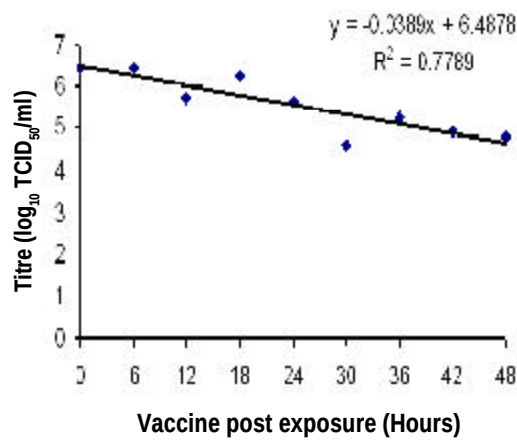
Fig. 16 : BPXV Diluted graphs at 45°C for LHT stabilizer



LHT+PBS 45°C



LHT+NSS, 45°C



LHT+MgSO₄ 45°C

LHT+DSW 45°C

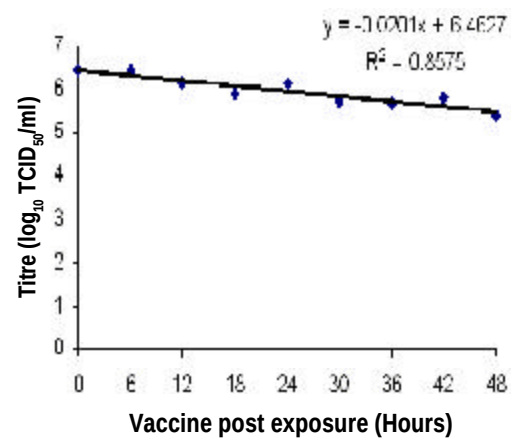


Fig. 17 : BPXV Diluted graphs at 4°C for TAA stabilizer

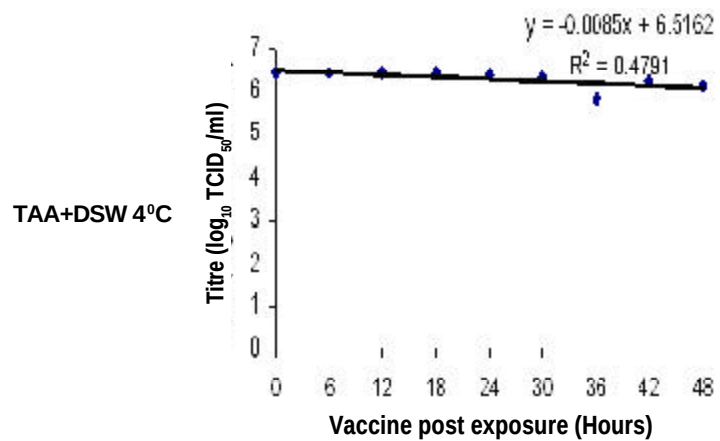
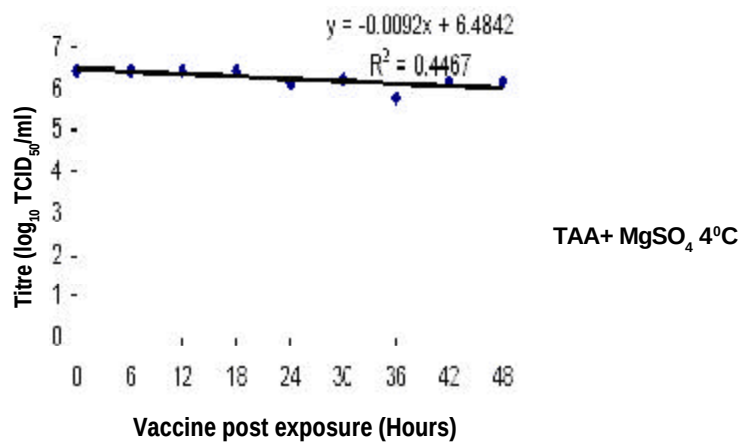
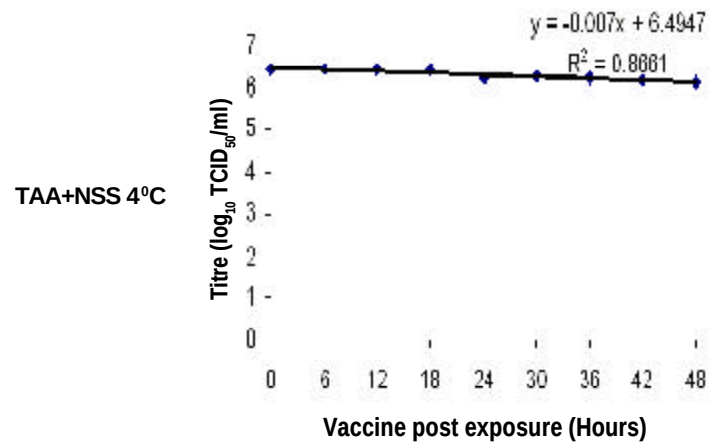
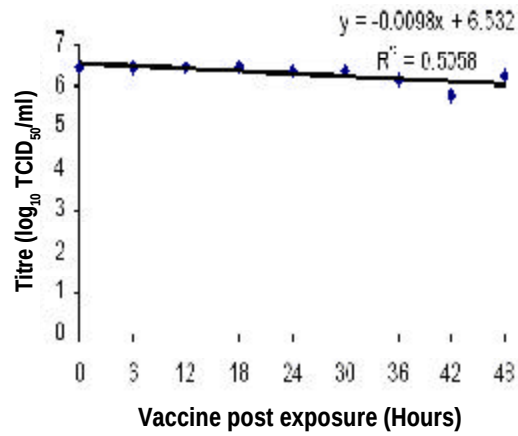
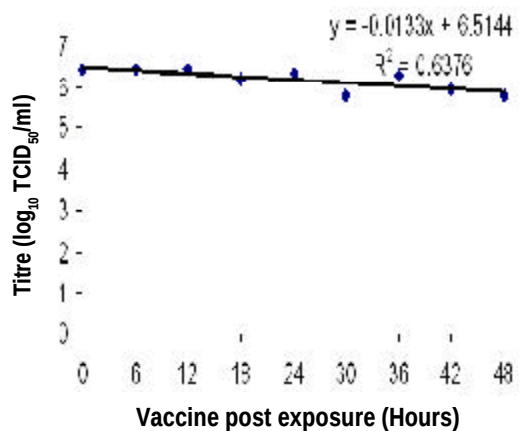
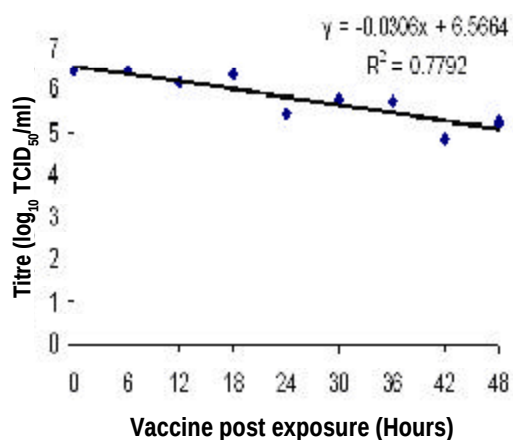
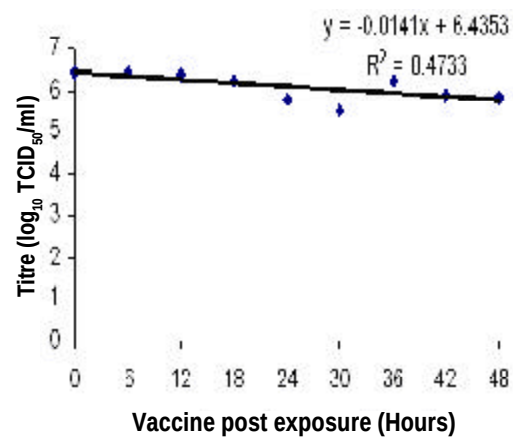


Fig. 18 : BPXV diluted graphs at 25°C for TAA stabilizer



TAA +PBS 25°C

TAA NSS 25°C



TAA + MgSO₄ 25°C

TAA + DSW, 25°C

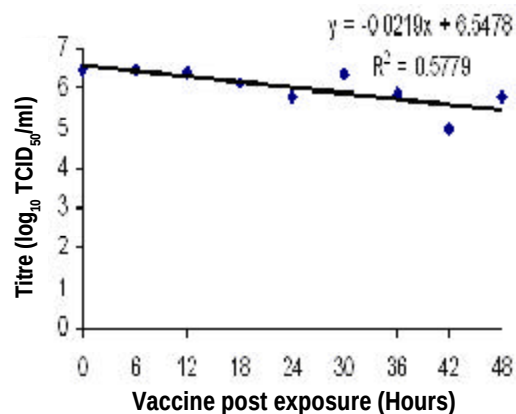
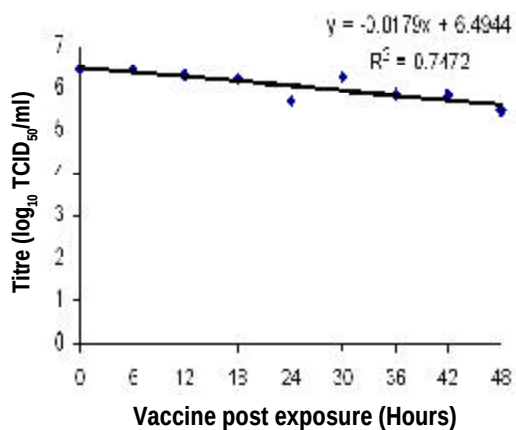
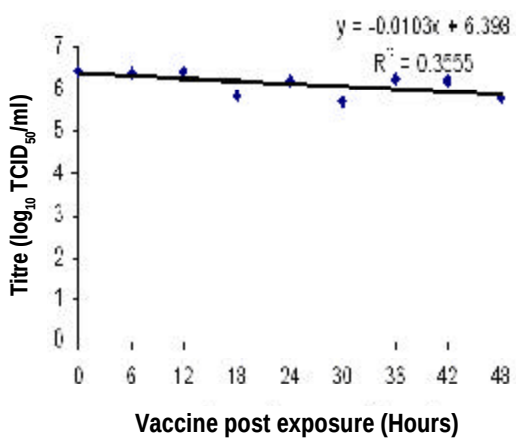
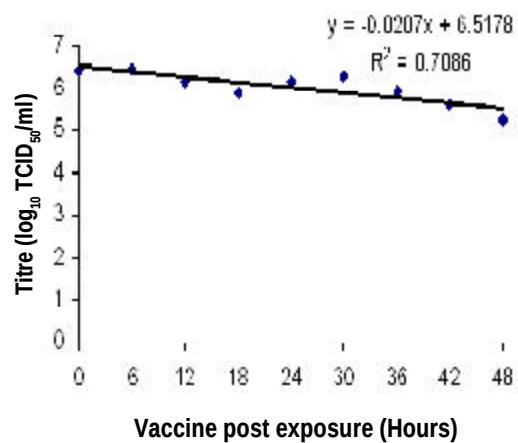


Fig. 19 : BPXV diluted graphs at 37°C for TAA stabilizer



TAA +PBS 37°C

TAA+NSS 37°C



TAA+MgSO₄, 37°C

TAA+DSW 37°C

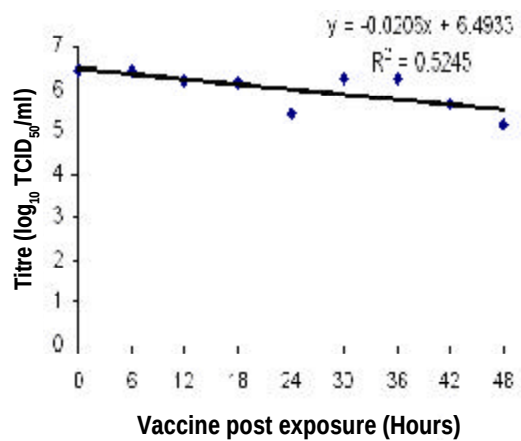
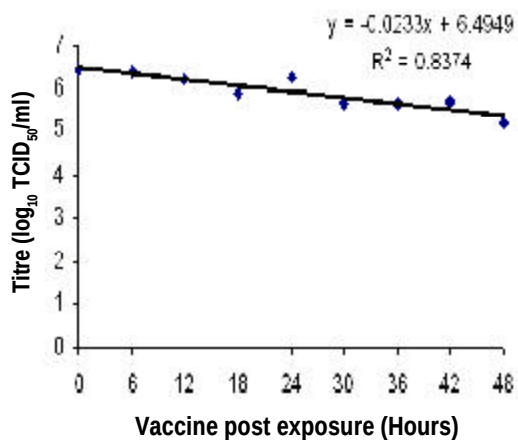
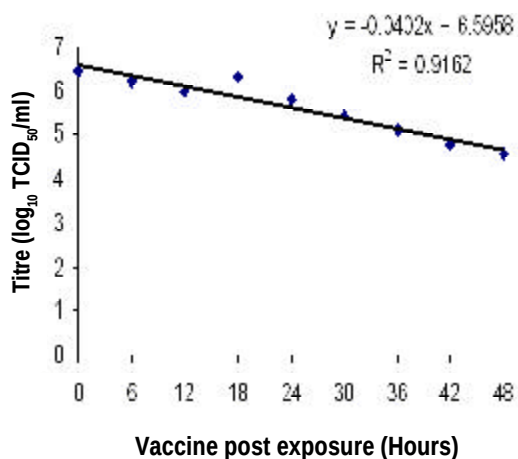
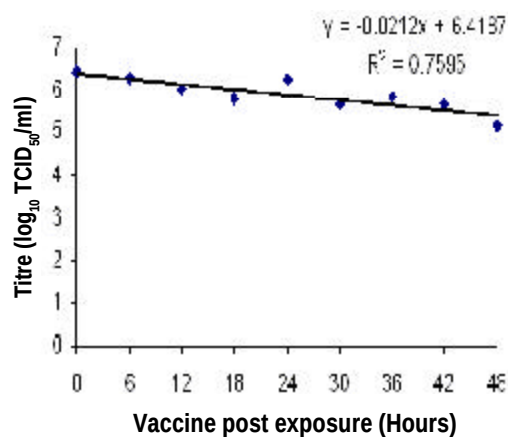


Fig. 20 : BPXV diluted graphs at 45°C for LHT stabilizer



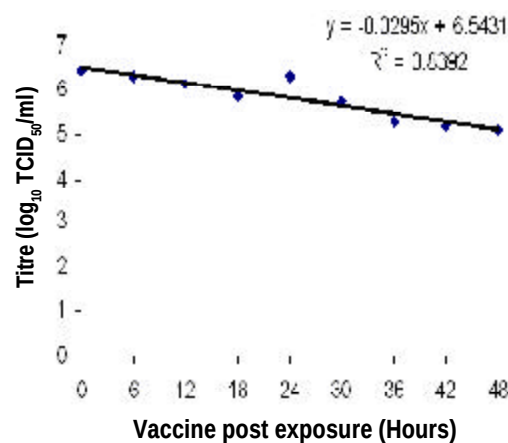
TAA + PBS 45°C

TAA + NSS 45°C



TAA + MgSO₄ 45°C

TAA + DSW 45°C



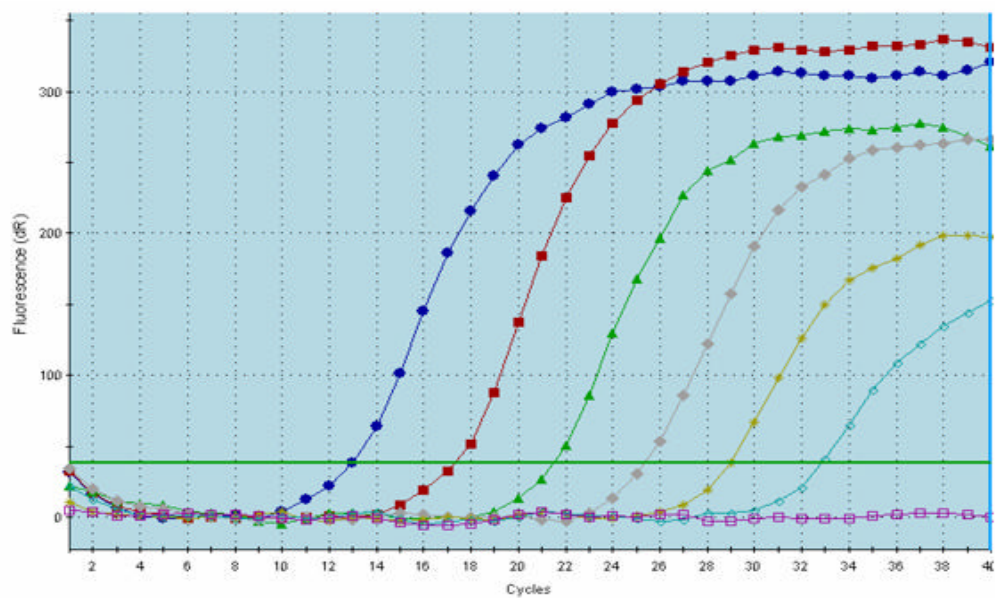


Fig. 21 : TaqMan probe based QPCR showing the amplification plots of DNA from different dilutions of reference vaccine batch at 30 hr harvest.

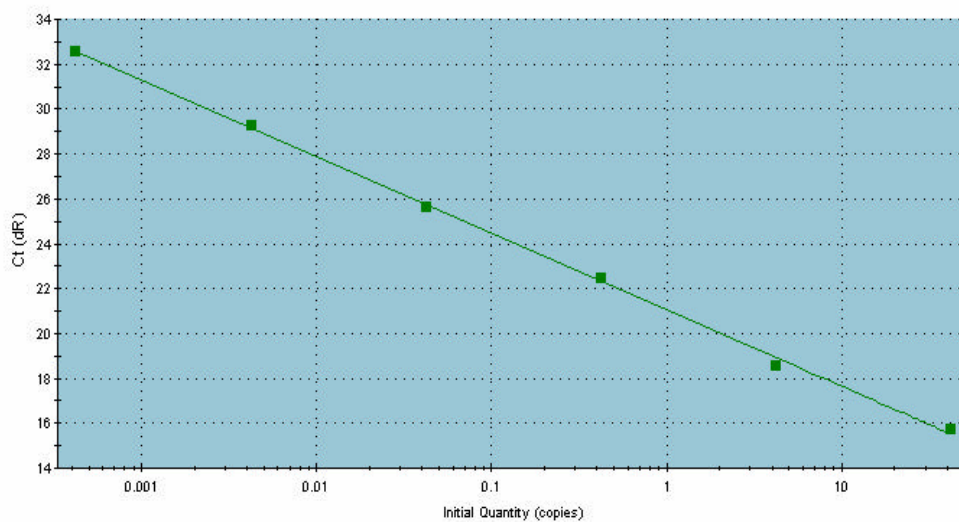


Fig. 22 : Standard curve established from different dilutions of the reference vaccine batch harvested at 30 h.

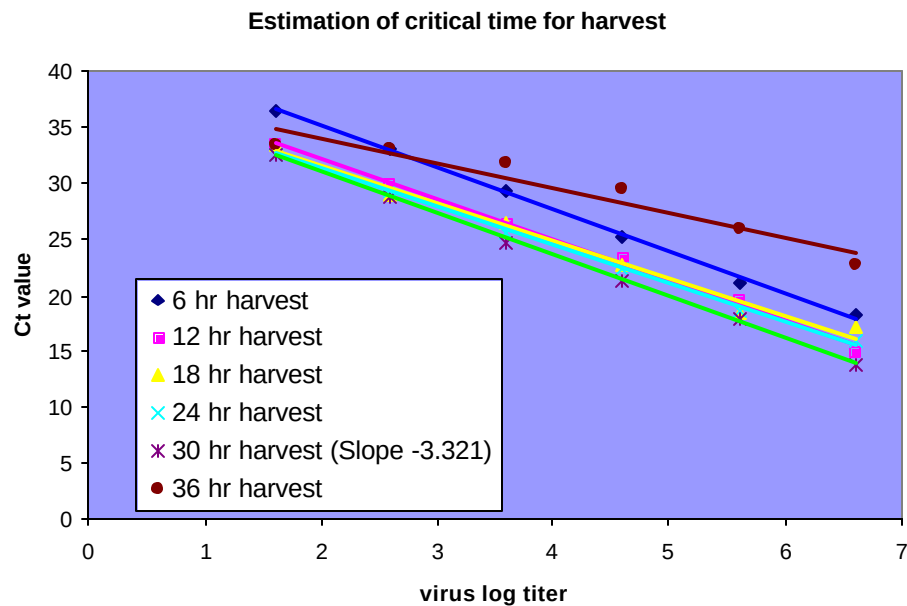


Fig. 23 : Standard curves established in TaqMan probe based QPCR using ten fold serially diluted (from neat to 10^{-4}) Bpxv vaccine virus P50 samples harvested at 12, 24, 30, 36, 36, 42 and 48 hours post infection into confluent Vero monolayer: the slope of each time interval of harvest are displayed, among all harvests the 30 hours post-infection is having the maximum slope, which is considered as the best time for harvest test and reference samples for assigning the titer.

4.8.1. Establishment of standard reference vaccine

Freeze dried BPV06/01 batch of live attenuated buffalopox vaccine, which was produced in-house at pox virus disease laboratory, Division of virology, IVRI, Mukteswar was selected for establishment of reference. The TCID₅₀ of the reference was calculated by Reed and Muench (1938) method by three consecutive titration in Vero cell line and found that the titre was $10^{6.62 \pm 0.32}$. The reference vaccine batch was stored at -20°C till further use. The titer of reference was rechecked at different time interval during storage to rule out any untoward effects of storage and found that there was not much change in the virus titer.

4.8.2. Establishment of critical time for harvest

4.8.2.1 TaqMan probe based Q PCR

The critical time to harvest reference vaccine was established by infecting the serially diluted BPXV 06/01 virus to 24h confluent Vero cell monolayer in the six well plates. The freeze-dried reference vaccine was reconstituted in EMEM medium and serially diluted in ten fold ranging from neat to 10^{-5} . The hundred micro liters (0.1 m.o.i.) of each dilution was inoculated to 24h confluent Vero cell monolayer in six well plates with Vero cell control. Each plate was harvested by freezing and thawing at 6, 12, 18, 24, 30, 36, 42 and 48h post infection. The DNA was isolated from each harvest were subjected to QPCR to establish the standard curves (Fig 21 & 22). Amplification plots are shown in Fig. 4. The slopes of the standard curves of the entire time interval harvest were compared (Fig.23). There was decrease in the slope of the standard curves as the time of harvest increases. Of all the time interval of harvest, the 30 h post infection had maximum slope. Hence, 30 h post infection was considered as critical time point for harvest for both the test and reference, to assign the comparative live virus titer for a given test batch of vaccine.

4. 8.2.2 Validation of vaccine batches

Six batches of attenuated buffalopox vaccine viz. **01/091209LS, (01/060110) TAA, (03/311209) LHT, 02/161209) LS, 03/311209 LS, 01/191209** and a reference batch of vaccine, **01/0609** was chosen for validation of QPCR. Twenty-four hour confluent Vero cells infected with tenfold serial dilution (neat to 10^{-5}) of all the of test batches and reference. The DNA isolated from the harvests at 30 h post infection was subjected to TaqMan probe based

QPCR and standard curves were established for the reference by plotting Ct values against $\log_{10}$ virus titres. The Ct values of test vaccine batches were compared with the standard curve of the reference to determine the titer of the test vaccine (Table 13-18). The results obtained were compared with titer calculated in the Vero cell monolayer by Reed and Muench(1938) formula and co-efficient of correlation was analyzed by student's t-test. The results of TaqMan probe based QPCR were found to be comparable (t- test values for TaqMan QPCR 0.27, at $p < 0.05$) and were within the acceptable limits.

Table 13-18**Batch 1(01/091209) LS**

Sl No	Dilution	Ct Value	Corresponding titre/ml	Avg titre/ml	TCID ₅₀
1	Neat	14.56	6.6025	6.6875± 0.095	6.54±0.042
2	10 ⁻¹	18.79	5.6125		
3	10 ⁻²	21.82	4.750		
4	10 ⁻³	24.36	3.60		
5	10 ⁻⁴	27.18	2.825		
6	10 ⁻⁵	31.08	1.735		

Batch 2 (01/060110) TAA

Sl No	Dilution	Ct Value	Corresponding titre/ml	Avg titre/ml	TCID ₅₀
1	Neat	15.33	6.425	6.328	6.30±0.18
2	10 ⁻¹	18.70	5.517		
3	10 ⁻²	22.55	4.30		
4	10 ⁻³	26.67	3.12		
5	10 ⁻⁴	29.81	2.10		
6	10 ⁻⁵	31.67	1.51		

Batch3 (03/311209) LHT**Batch 4 (02/161209) LS**

Sl No	Dilution	Ct Value	Corresponding titre/ml	Avg titre/ml	TCID ₅₀
1	Neat	13.93	6.80	6.568	6.42±0.25
2	10 ⁻¹	17.03	5.80		
3	10 ⁻²	20.93	4.75		
4	10 ⁻³	25.84	3.25		
5	10 ⁻⁴	28.49	2.50		
6	10 ⁻⁵	32.03	1.310		

Batch 5 (03/311209) LS

Sl No	Dilution	Ct Value	Corresponding titre/ml	Avg titre/ml	TCID ₅₀
1	Neat	13.35	6.812	6.729	6.64±0.20
2	10 ⁻¹	17.39	5.810		
3	10 ⁻²	22.03	4.810		
4	10 ⁻³	24.36	3.432		
5	10 ⁻⁴	28.10	2.825		
6	10 ⁻⁵	31.56	1.687		

Batch 6 (LS)

Sl No	Dilution	Ct Value	Corresponding titre/ml	Avg titre/ml	TCID ₅₀
1	Neat	13.51	6.790	6.573	6.52±0.16
2	10 ⁻¹	18.09	5.770		
3	10 ⁻²	22.14	4.710		
4	10 ⁻³	25.20	3.50		
5	10 ⁻⁴	29.07	2.270		
6	10 ⁻⁵	31.82	1.40		



Buffalopox is a highly contagious, economically important viral disease, not only of buffaloes and cows but also a zoonotic infection of human beings specially in milking attendants. The causative agent of buffalopox, buffalo pox virus (BPXV), is classified in the *Orthopoxvirus* (OPV) genus of the subfamily *Chordopoxvirinae* and the family *Poxviridae* (Von Regenmortel *et al.*, 2000). The disease is enzootic in India and economically important one as it causes severe morbidity, mortality and productive loss in affected animals. Outbreaks of buffalopox are reported not only from India but also from many other countries including Pakistan, Egypt, Nepal and Bangladesh. Recently, similar outbreaks of pox-like-infections caused by Vaccinia virus (VACV)-like agents viz. Cantagalo (Damaso *et al.*, 2000) and Aracatuba viruses (de Souza Trindade *et al.*, 2003) have been reported from Brazil. The WHO Joint Expert Committee on Zoonosis (FAO, 1967) has declared buffalopox as to be one of the important zoonotic diseases caused by a virus, closely related to VACV and frequently encountered in countries where buffaloes are reared as milch animals.

In countries where the disease is endemic and animal movement is difficult to restrict, disease control becomes complex. The problem is compounded by lack of precise diagnostics and prophylactics. In such circumstances, one of the effective means of containing the spread of infection is employing sanitary measures such as segregation of infected animals. Recently a live attenuated vaccine has been developed using Vero cell-adapted BPXV (VIJ/96, P-50) and compared (Personal communication) with the already available reference strain (BP4) vaccine. The vaccine is currently undergoing extensive field trials in different parts of the country. Therefore, in the present study, the new vaccine was evaluated for its thermo stability and potency.

India being a subtropical country, it experiences wide range of temperatures. Therefore maintenance of shelf-life of the vaccine is difficult and needs cold chain. Like many other modified live attenuated lyophilized vaccines, the attenuated buffalo pox vaccine is no exception in thermal inactivation. The vaccine requires cold chain for storage and transport. As a result, the cost of production and transportation are high and user's expenses are unavoidable, especially in developing tropical and semitropical countries like India. Therefore, evaluation of the vaccines for its thermostability with different stabilizers is of much demanding in order to maintain immunization effectiveness through reduction of potency loss due to heat damage; reduce cold chain costs and logistical requirements.

Vaccine quality

Taking the field conditions of our country in to consideration, this study was envisaged to compare the efficacy of three different stabilizers (LS, LHT and TAA) in the induction of thermal protection to the candidate buffalopox virus at various temperatures normally encountered at field conditions. The vaccines were freeze-dried in batches with different stabilizers. Under these freeze-drying conditions, there was a loss of very little titer and the range was within the acceptable limit.

Residual moisture (RM) in a vaccine also contributes to vaccine quality. In the present study, RM ranged from 3.25 to 5.20 %, which is considered to be relatively high. High RM may be due to the absence of heating phase of the vaccine vials during secondary drying.

Stability of the freeze-dried vaccine

The major disadvantage associated with live attenuated vaccine is the maintenance of 'cold-chain' which is a very costly affair. Therefore, it is desirable for an ideal stabilizer to maintain recommended dose of $5\log_{10}$ TCID₅₀/100 doses in a vaccine vial as per OIE for a period as-long-as possible at a given temperature.

Although thermo stability studies on different other viral vaccines has been exhaustively studied(Plowright, 1963; Leiss and Plowright, 1963; DeBoer and Barber, 1964; Anon, 1966; Plowright *et al.*, 1970, 1971; Bansal *et al.*, 1974; Languet *et al.*, 1985; Mariner *et al.* 1990a; Sarkar *et al.*, Riyesh *et al.*,) no such studies in respect of buffalopox vaccines are conducted. In this study, the vaccine formulations were made using the vaccine viruses developed at IVRI,

Mukteswar at a passage level of P-50 which has been studied for its innocuity, safety and efficacy. Three stabilizer formulations were employed for this vaccines viz., (i) Lactalbumin hydrolysate (LAH) + sucrose (LS) and (ii) Lactalbumin hydrolysate (LAH) + Trehalose di hydrate (LHT) as reported for TCRP vaccine (Mariner *et al.*,1990), and (iii) Stabilizer TAA (Trehalose+ Amino acids with divalent cations (TAA) (Adebayo *et al.*,1998).

At 4°C all the three stabilizer combinations were found to maintain the required infectivity titer during the entire period of study. This temperature is generally used for short-term storing of the vaccines .However, vaccines with LS stabilizer showed better stability over other two stabilizers in terms of its shelf-life and half-life.

At 25°C, stabilizer LS showed better stability compared to the LHT and TAA with a high shelf life as well as half life. At temperature 37°C, the one which is normally encountered in many parts of the country during summer months (March-July), vaccines prepared in the present study using LHT stabilizer showed comparatively better stability than to other two stabilizers with a shelf life and half life of 31.16 and 6.1 days respectively. The results indicate that the LHT stabilized vaccine has marginal stability at ambient temperature compared to LS stabilizer. However, TAA stabilizer was found relatively not suitable for buffalopox vaccine at this temperature.

At 45°C, two stabilizer combinations, LS and LHT showed stability up to 36 hours while, TAA stabilizer could maintain the titer up to 24 hr only after which the titer started to receding. At this temperature, the shelf-life and half-life of the vaccine in LS stabilizer found advantageous over other two.

Other factors which could be implicated in the poor performance of our vaccine at 25°C, 37°C and 45°C may be due to the presence of higher RM. Influence of residual moisture (RM) on the viability of the vaccines has been reported earlier (Precausta *et al.*, 1982;Mariner *et al.*,1990). Therefore, it has been suggested to extend the secondary drying to ensure low RM as it was having detrimental effect on viral vaccine (Worrall *et al.* 2001). In studies conducted for TCRP (Mariner *et al.*, 1990) and PPR (Worrall *et al.*, 2001) vaccines, indicated that the maximum permissible RM should be below 2.5%. It has been reported that increasing the length and final temperature of lyophilization cycle will have a suitable effect on the stability

of the vaccine as it reduces the RM without affecting the infectivity titres. However in the current study, freeze-drying was carried out using a conventional freeze-drier in which the cycle length was 48 hrs and final temperature was -60°C and the secondary drying was carried out without the application of heat during drying process. This might have caused high RM of all the freeze-dried vaccine and thereby reducing their stability.

Earlier studies for TCRP and PPR vaccines have indicated that the increase in the concentration of trehalose dihydrate increased the stability of the vaccine (Worrall *et al.*, 2001). In contrary in the present study, stabilizer TAA high trehalose did not show good result in any of the temperatures studied.

Reconstituted vaccines

Except for few cases the titre was more than the recommended dose for a 100 dose vial ($5 \log_{10} \text{TCID}_{50}$). This titre was maintained even up to 48 hrs both at 4°C and 25°C with all the four diluents used. But at 37°C and 45°C , the diluents NSS and PBS performed well. 1M MgSO_4 diluent could not maintain the titer even at lower temperatures. The vaccine reconstituted with NSS can be used at ambient temperatures even up to 48 hrs and possibly more. Hence, this study confirms NSS as a diluent of choice for buffalopox vaccine.

Potency by real time PCR

In general, efficacy of vaccine and seroconversion depends on the number of live virus particles in attenuated vaccine. Therefore, assignment of number of live virus particles per dose, is the important aspects of quality control. Most of the virus quantitation methods are based on end point dilution methods, which rely on virus-induced cytopathology. These include, Reed and Muench (1938) end point dilution method or plaque assay for quantitation of infectious virus particles (Gaush and Smith, 1968; Smith *et al.*, Sood *et al.*, 1995). Even though these assays are considered to be the gold standard, they have certain drawbacks, which impede them from long-term implementation for estimation of titer in live attenuated vaccine. These assays are basically a subjective interpretation; hence there will be variation in the assigned titer between laboratory to laboratory, person to person and from time to time. For instance, in one of the study carried out by Fukuda *et al.* (1996), minor modifications in the procedure for plaque assay resulted in differences of potency values up to $1 \log_{10}$ PFU/ dose. A study

involving 38 laboratories showed a median variation of $2 \log_{10}$ PFU/ dose between the estimates using PFU and titration of the measles vaccine virus (Forsey *et al.*, 1992). In a similar study involving ten laboratories, the variation to the extent of $1 \log_{10}$ for measles virus in the trivalent vaccine of measles, mumps and rubella has been reported (Forsey *et al.*, 1993). Thus, these assays suffer from disadvantages like labor intensivity, time consumption, throughput restriction and variability. These traditional assays are not the preferred tests of choice to assign titer to the individual components of bivalent or multivalent vaccines. Titration would necessitate a highly specific neutralizing antibody against each component of multivalent or combined vaccine, which however is not fullproof method as cent percent neutralization is highly unlikely.

Over recent years, quantitative polymerase chain reaction (QPCR) has been applied for estimating number of live virus particles in the monovalent (Wang *et al* 2005), bivalent or multivalent vaccines (Schalk *et al.*, 2004; Schalk *et al.*, 2005; Kallesh *et al.*, 2009). QPCR is based on kinetic fluorescence detection of the amplified products during PCR and is used to achieve accurate quantification of a DNA or RNA template. A linear correlation exists between the $\log_{10}$ of the template concentration ($\log_{10}$) and PCR cycle at which fluorescence raises above the background (*Threshold cycle, Ct*). Using the standard DNA or RNA of known concentration, the template DNA or RNA concentration (of both infectious and non-infectious virus) of an unknown sample can be quantified. However, in live virus vaccine preparation, all the genome measured by the QPCR is not necessarily infectious; hence PCR measure does not correspond to infectious load. To overcome this drawback, cell cultures are infected with vaccine sample and harvested at critical time point for QPCR. And as expected, only live viral particles can infect the cells. It is assumed that, if the content of the live vaccine vial is first propagated in cell culture, the amount of genomic DNA propagated in the cell culture corresponds to amount of viable virus present initially in the preparation. QPCR method designed to estimate the infectious virus load has been developed for measles vaccine, rotavirus in pentavalent rota virus vaccine, Rota teq® (Ranheim *et al.*, 2006), multivalent measles, mumps and rubella vaccine (Schalk *et al.*, 2004; Schalk *et al.*, 2005) and to assign infectious potencies to adenovirus based vaccine and vectors for gene therapy (Wang *et al.*, 2005). This approach is also applied for quantifying the gene therapy vectors (Shastry *et al.*, 2002).

One of the most important advantages of QPCR based estimation of titer is that the titer of vaccine can be estimated long before the CPE appears and this will save lot of time especially in case of the viruses which are known to produce delayed CPE. In case of buffalo pox viruses, the CPE starts to appear on the second day post infection and complete CPE appears at the end of third day. We applied QPCR based technique by which we can determine the titer of buffalo poxvirus in a given batch of vaccine as early as 30-h post infection, which drastically reduced the manpower and time required. At the same time assay is technically simple, specific, sensitive, reproducible and applicable to estimate the titer of the individual components in bivalent or multivalent or combined vaccines. Results obtained are comparable to the conventional methods of titer estimation.

The titre of standard reference vaccine virus was found to be $10^{6.62 \pm 0.32} \text{TCID}_{50} / \text{ml}$. Selection of the vaccine batch is based on the assumption that the titres of the test batches may fall within the wide range of these reference vaccine dilutions. In other words, it is expected that the Ct values of any dilutions of test vaccine virus would fall in the range of Ct values of reference. This would enable minimum number of dilutions of test vaccine batches for estimation of virus titre.

Further, the critical time point of harvest was determined for cells post infection to estimate the live viral particles in given test vaccine batch. The critical time point for harvest corresponds to the minimum time required for appearance of sufficient viral particles in all the dilutions of buffalopox virus infected. The TaqMan probe based QPCR was carried out with the cells harvested at 12, 18, 24, 30, 36, 42 and 48h post infection. The experiment was repeated thrice and average slope of standard curves for each interval of harvest was compared. The results showed that there is gradual decrease in slope of standard curve with increase in duration of infection. The slope of the standard curves tended to flatten with increasing time of harvest, implying unavailability of the healthy Vero cells as host for newly replicating buffalopox virus in those wells that received less diluted virus. The slope of standard curve for 24, 30 and 36h post infection were found to be relatively comparable. However, the 30-h post infection was found to have maximum slope (3.312). Thus, this particular time point was considered as the critical time point to harvest the reference as well as test vaccine batches so as to get maximum live viral particles.

For validation of QPCR assays, six batches of live attenuated buffalopox vaccine including a reference buffalopox vaccine (Batch-31/12) were selected to estimate the titer of buffalopox vaccine. The results obtained were found to be satisfactory and within the acceptable range of accuracy. High degree of correlation (0.9648) was observed between the titres estimated by the QPCR and conventional infectivity assay with no significant difference ($p < 0.005$) noticed between the methods. These findings are in agreement with earlier findings (Kallesh *et al.*, 2009).

Conclusions

- Among the three stabilizers used for preparation of buffalo pox vaccine, LS stabilizer was the best choice for buffalopox vaccine.
- Among the four diluents studied for reconstitution of the buffalopox vaccine, NSS showed better result in all conditions.
- The TaqMan probe based QPCR was successfully employed to estimate the titer of buffalopox virus in attenuated buffalopox vaccine. The critical time point of harvest was determined as 30h post infection to estimate buffalopox virus load.
- The titers obtained in QPCR assays and Reed and Muench (1938) end point dilution method ($TCID_{50}$) were relatively comparable with each other without any significant difference ($p \leq 0.005$).
- Further validation of the QPCR is warranted to attribute repeatability, reproducibility to this novel and quick method of potency estimation for its wider application.

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Buffalopox is a highly contagious, economically important viral disease, not only of buffaloes and cows but also a zoonotic infection of human beings specially in milking attendants. The disease is enzootic in India and economically important one as it causes severe morbidity, mortality and productive loss in affected animals. Outbreaks of buffalo pox are reported not only from India but also from many other countries including Pakistan, Egypt, Nepal and Bangladesh. Recently, similar outbreaks of pox-like-infections caused by Vaccinia virus (VACV)-like agents viz. Cantagalo and Aracatuba viruses have been reported from Brazil. Presently no suitable vaccine is available in our country for control of this dreadful disease. Recently, in our laboratory, a live attenuated vaccine has been developed using Vero cell-adapted BPXV (VIJ/96, P-50) and compared with the already available reference strain (BP4) vaccine. The vaccine is currently undergoing extensive field trials.

In the present study, live attenuated BPXV vaccine was evaluated for its thermostability using three different stabilizers by exposing them to different temperatures like 4°C, 25°C, 37°C and 45°C both in lyophilized as well as in reconstituted form. Vaccines prepared with LS stabilizer showed better stability compared to other stabilizers. However, at 37°C LHT stabilizer was found to be superior over other two stabilizers.

For reconstitution of the vaccines four different diluents were used viz, PBS, NSS, 1M MgSO₄ and DSW and exposed to various temperatures like 4°C, 25°C, 37°C and 45°C and titrated every 6hr intervals up to 48 hr. Vaccine diluted with NSS showed better stability in all situations by maintaining minimum required titer over the entire sampling period. Diluent PBS and DSW showed comparable results. 1M MgSO₄ could not maintain the required titre with any of the stabilizer combinations at higher temperatures .

In general, efficacy and seroconversion of a vaccine depends on the number of live virus particles. Therefore, to assign the number of live virus particles per dose in each batches of attenuated vaccine is one of the important aspects of quality control of attenuated vaccines. Most methods of virus quantitation are based on the end point dilution method, they rely on virus-induced cytopathology. These include, Reed and Muench or plaque assays for quantitation of infectious virus particles. These assays are basically a subjective interpretation, hence there will be variation in the assigned titer between lab to lab, person to person and from time to time. Even though these assays are considered to be the gold standard methods, some of the drawbacks as mentioned previously precludes them from long-term implementation for estimation of titer in live attenuated vaccine. Recently QPCR has been used as novel approach for the potency estimation of either monovalent or multivalent vaccines for its advantages viz. high sensitivity, specificity, reproducibility and rapidity. The QPCR is very flexible without need for post PCR manipulations. In the present study, the estimation of live buffalopox virus particles in the attenuated buffalopox vaccines using quantitative polymerase chain reaction (QPCR) as an alternative approach to conventional end point titration ($TCID_{50}$) method was investigated

One of the live attenuated BPXV vaccine batch was selected as standard reference vaccine and its titer was assigned by Reed and Muench end point titration method in Vero cell line. The titer of this reference vaccine was found to be $10^{6.62}TCID_{50}/ml$ in three consecutive experiments. The reference vaccine vials were stored at $-20^{\circ}C$ till further use. The reference vaccine virus was diluted serially ten fold followed by infection to 24 h confluent Vero cell monolayer and harvested at 6,12, 18, 24,30,36, 42 and 48h post infection. The DNA isolated from all intervals of harvests were subjected to TaqMan probe based QPCR for determination of the critical time point to estimate live virus particles in the given test batch of vaccine. The standard curves were established using the data obtained from three consecutive experiments. The slope of the standard curves of each interval of harvest with different dilutions of reference were compared and slope of the curves were determined. The 30 h harvest was giving maximum slope (-3.312) and hence was considered as the critical time point for harvesting the test vaccine batches.

For validation of the QPCR assays, six buffalopox vaccine batches produced in Vero cells using roller cell culture system were selected (03/311209) ,(01/060110) and (01/

091209) along with the reference batch of vaccine (batch 03/311209). All the batches of vaccine virus were serially diluted, ranging from neat to $10^{-5} \log_{10}$ dilutions and infected onto 24 h old confluent Vero cell monolayer, followed by harvesting at 30 h post infection. The QPCR was carried out with the DNA isolated from all the harvest. The standard curve was established for the reference batch of vaccine by plotting the threshold cycle (Ct) values against the log titer dilutions. The Ct values of the test vaccine batches were compared with the standard curve of reference and the titers were assigned. The titer obtained by QPCR and the endpoint dilution method ($TCID_{50}$) were comparable to each other as analyzed by student's t-test. The titers obtained by QPCR and the endpoint dilution ($TCID_{50}$) method were again comparable to each other as analyzed by student's t-test and was within the acceptable range.

Thus, the QPCR assay could be explored for its potential to replace or to use it in tandem with conventional method of end point titration for assaying the live buffalopox virus load in a given batch of buffalopox vaccine. Nevertheless, further validation of the QPCR is warranted to attribute repeatability to this novel and quick method of potency estimation for its widespread application.



In the present study, we have focused on the thermo-stability and potency of live attenuated buffalo pox vaccine prepared at I.V.R.I Mukteswar. BPXV vaccine was evaluated for its thermo-stability using three different stabilizers by exposing them to three different temperatures like 4°C, 25°C, 37°C and 45°C both in lyophilized as well as in reconstituted form. Vaccines prepared with LS stabilizer showed better stability compared to other stabilizers. For reconstitution of the vaccines four different diluents were used viz, PBS, NSS, 1M MgSO₄ and DSW and exposed to various temperatures like 4°C, 25°C, 37°C and 45°C and titrated every 6hr intervals up to 48 hr. Vaccine diluted with NSS showed better stability in all situations by maintaining minimum required titre over the entire sampling period. Diluents PBS and DSW showed comparable results. 1M MgSO₄ could not maintain the required titre with any of the stabilizer combinations at higher temperatures.

In general, efficacy and sero-conversion of vaccine depends on the number of live virus particles in attenuated vaccines, therefore, live virus particles per dose is one of the important aspects of quality control. The application of quantitative polymerase chain reaction (QPCR) has now been exploited for the potency estimation of various monovalent or multivalent vaccines for its advantages. In the present study, the QPCR for estimation of live buffalopox virus (BPXPV) particles in the monovalent. A batch of buffalopox vaccine, the titre of which was calculated by TCID₅₀ method was selected as reference. A critical time point for harvest of the Vero cells infected with various log₁₀ dilutions of reference virus was determined as 30 h, by comparison of the slopes of standard curves established with harvests of different time 6 hrs intervals. The QPCR was carried out using DNA isolated from Vero cells harvested at 30h post infection with various dilutions of reference and test batches of vaccine viruses. The standard curve was established for the reference by plotting the threshold cycle (Ct) values against the log₁₀ titer dilutions and the titer of test batches was calculated by comparison of its Ct values with that of reference. The titers estimated by QPCR and TCID₅₀ methods were well comparable to each other as analyzed by student's t-test with out any significant difference (P<0.05). The assay thus could be used as a quick alternative for estimation of live BPXV particles in either buffalopox vaccine. Further validation of the TaqMan based QPCR is warranted to attribute accuracy, repeatability, reproducibility in potency estimation for its wider application.

प्रस्तुत अध्ययन में भैंस का चेचक विषाणु के प्रतनु टीके का उष्ण-प्रतिरोधकता और सामर्थ्य के उपर केन्द्रित किया है जोकि अर्पीआर मुक्तेश्वर में तैयार किया गया। तीन अलग-अलग स्टेविलाइजर का उपयोग टीके को बनाने में किया गया और इसे चार अलग-अलग तापमान पर रखा गया जैसे कि 4°C , 27°C , 37°C , 45°C टीका जोकि एल.एस. स्टेविलाइजर के उपयोग से घोला गया, अन्य से ज्यादा अच्छा परिणाम दिया, चार विभिन्न घोलक पदार्थ जैसे PBS, NSS, MgSO_4 , DSW और पानी और अलग-अलग तापमान पर रखा गया जैसे, 4° , 25° , 37° , 45°C और प्रत्येक छः घण्टा के अन्तराल पर टाइटेड किया गया। इनमें से NSS द्वारा घोला गया टीका ने सबसे अच्छे परिणाम दिया जबकि PBS और जल ने सामान्य परिणाम दिखाया और MgSO_4 ने उन्नत उष्णता में अच्छे टाइटर नहीं बना रख पाये।

सामान्य, टीके का सामर्थ्य एवं लसी-रूपान्तरण प्रतनू टीके में उपस्थित सजीव विषाणु लव निर्भर करता है, इसलिए सजीव विषाणु निरूपण करना टीका प्रकृति अभिकार का अति महत्वपूर्ण रूप है। आजकल मात्मिक पुरुभाजन श्रृंखला प्रतिक्रिया इसके लाभों के कारण, एक संयुज अथवा बहुयुक्त टीके के शक्ति मापन के लिए समुपयोजित होती है। प्रस्तुत अध्ययन में, टेक मैन एषणी आधारित प्रक्रिया को टीके के सामर्थ्य ज्ञात करने के लिए उपयोग किया गया। एक भैंस चेचक टीके का दल, जिसका इमदीश्य रीड एवं मुंच विधि के द्वारा निर्धारित किया गया, को सन्दर्भ नमूने के रूप में चुना गया। विभिन्न समय अन्तराल के सस्य द्वारा स्थापित मानक वक्रों के ढाल, तलों की तुलना करके विभिन्न $\log_{10}$ डाइलुशन्स द्वारा संक्रमित विरो कोशिका के सस्य के लिए काष्ठकाल को तीस घंटे मापा गया। सस्य विरो कोशिका के विभिन्न सन्दर्भ टीका डाइलुसन एवं परीक्षण टीके के डी.एन. ए. से मात्रात्मक पुरुभाजन श्रृंखला प्रतिक्रिया की गई। देहली चक्र माताओं को $\log_{10}$ इयदीक्ष्य के प्रतिकूल लेकर सन्दर्भ टीका दल के लिए प्रमाण वक्र स्थापित किया गया। जैसा कि विद्यार्थी टी-टेस्ट से विश्लेषित हुआ है, मात्रात्मक पुरुभाजन श्रृंखला प्रतिक्रिया एवं अन्तिम विन्टु डाइलुसन द्वारा प्राप्त इयदीक्ष्य एक दुसरे के तुलनात्मक है। अतः यह परीक्षण भैंस चेचक टीके में संजीव विषाणु मापने के लिए एक तेज विकल्प के रूप में प्रयोग हो सकता है। आगे, शक्तिमापन में पुनुरावृत्ति एवं पुनुरुत्पादित उपरोपन के लिए अधिक वैधकरण अधिप्रव है।

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Appendix

Cell culture media and buffers

Eagle's MEM (1 Liter)

EMEM powder (Sigma)	1liter bottle
NaHCO ₃ (7.5%)	10 ml
Glutamine (3.0%)	10 ml
Antibiotic stock	10ml

Dissolve the EMEM powder in 1 liter of distilled water and autoclave at 120°C at 15lb pressure for 10 minutes. Keep at 4°C till use. Glutamine (3%), NaHCO₃ (7.5%) and antibiotic solutions were added in media just before use.

Antibiotic stock

Benzyl Penicillin (10,00000U)	5 vials
Streptomycin (1gm/vial)	5vials
Autoclaved PBS	500ml

Trypsin-Versene (100ml)

Versen	0.700gm
D-Glucose	1.000gm
KCl	0.400gm
NaCl	8.000gm
NaHCO ₃	0.580gm
Trypsin	1.000gm
Phenol red	0.002g,
Distilled water	up to 100ml

Dissolve the contents in distilled water and sterile through filtration (Seitz filter).

Hank's balanced salt solution (HBSS)

NaCl	8.000g
KCl	0.400g
KH_2PO_4	0.060g
$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$	0.057g
Phenol red	0.011g
DW up to	1000 ml

pH 7.4

Sterilization by autoclaving at 121°C at 15 lb for 15 minutes

Stock buffers for molecular biology

Luria Bertani Broth

Tryptone	10 gm
NaCl	5 gm
Yeast Extract	5 gm

Distilled Water up to 1 Liter

The pH was adjusted to 7.5 using 10N NaOH and the n sterilized by autoclaving.

To prepare LB Agar, 1.5% agar was added to LB medium. Where indicated ampicillin was added to the medium @100µg/ml.

LB plate with ampicillin , IPTG, X-Gal

Sterile LB agar was cooled down to about 50°C and added with ampicillin to a final concentration of 50µg/ml and IPTG to a final concentration of 50µg/ml and X gal to final concentration of 50µg/ml. the mixture was poured on to the sterile petridish @ 15 ml per plate and allowed to solidify

SOB Medium

Tryptone	2 gm
NaCl	0.05 gm
KCl	0.0186 gm

2M Mg ⁺⁺	1.0 ml
Yeast Extract	0.5 ml
Distilled Water	up to 100 ml

SOC Medium

SOB Medium	980 ml
1M Glucose	20 ml

X-Gal (100X) (5-bromo-4-Chloro-3-indolyl-B-D-galactopyranoside)

X-gal	25 ml
N’N’ Dimethyl formamide	1 ml
Stored at -20°C	

IPTG (100X)

IPTG	25 gm
Distilled water	1 ml
Sterilized by filtration	

Ampicillin Stock

Ampicillin (Sodium Salt)	150 gm
Distilled Water	up to 100 ml

TSS (Transformation and Storage Solution)

2X LB Medium (pH 6.5)	50 ml
30% PEG (8000) with 60mM Mg ⁺⁺	5 ml
Adjust pH to 6.5	