Q-PCR BASED POTENCY ESTIMATION AND THERMO-STABILITY EVALUATION OF LIVE ATTENUATED CAMELPOX 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

DEEMED UNIVERSITY

INDIAN VETERINARY RESEARCH INSTITUTE

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2010

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Certified that the research work embodied in this thesis entitled, **"Q-PCR based potency estimation and thermo-stability evaluation of live attenuated camelpox vaccine"** submitted by **Dr M Prabhu** (Roll No. 4739) for the award of the degree of **Master of Veterinary Science** of Indian Veterinary Research Institute, is the original work carried out by the candidate himself under my supervision and guidance.

It is further certified that **Dr M Prabhu** has worked for more than 20 months in the institute and has put in more than 150 days attendance under me, from the date of registration for the degree of **Master of Veterinary Science** of the University as required under the relevant ordinance.

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Certified that the thesis entitled **"Q-PCR based potency estimation and thermo-stability evaluation of live attenuated camelpox vaccine"** submitted by **Dr. M Prabhu** (Roll No. 4739) in partial fulfillment of the requirements for the degree of **Master of Veterinary Science** of Indian Veterinary Research Institute, embodied the original work done by the candidate. The candidate has carried out the work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by him for the award of the degree of Master of Veterinary Science of Indian Veterinary Research Institute.

It is further certified that the candidate has completed all the prescribed requirements governing the award of the degree of Master of Veterinary Science of Indian Veterinary Research Institute.

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Acknowledgements

The pleasant journey in the pursuit of my M.V.Sc. degree was far more than educational; it enriched my life and broadened my perspectives. Along this experience I was lucky enough to be guided by Dr V Bhanuprakash, whose advice always came with a friendly smile but also loaded with high expectations that helped me get to where I am today. I thank him for sharing all his knowledge with me and for giving me the opportunity to learn from expert researchers from our field.

Ever since I started in my lab it was Dr. V Gnanavel, who very patiently answered all my questions, introduced me to new techniques and listened to my complaints when things did not turn out as expected. I am grateful to have shared with him all these days in the lab. I would also like to thank my lab-mates and staff at the Pox Virus Disease Laboratory, who also were always there to help me during my learning process and research.

Achieving this goal was possible, thanks to Dr. A.B. Pandey, Head, Division of Virology, IVRI. I also extend my gratitude to the members of my advisory committee Dr. Balamurugan and Dr. Dandapat for offering their guidance and advice when I needed it.

Also, I would like to thank all beloved scientists at other laboratories of my Division for facilitating the access to all the needful things and for sharing important reagents and techniques. Essential contribution was provided by scientists of PD-FMD at different stages of my research work, for which I particularly thank Dr. Pattnaik.

I take the privilege to thank the honorable director and the venerable joint Director, IVRI for providing me a great opportunity to study in this reputed institution.

Nothing that I could write in this page could possibly reflect my endless gratitude to my family. Amma, Appa, Akka and my relatives thank you for giving me all that I needed and more, not only physically but morally and spiritually.

All my friends, this manuscript would not have been the same without your unconditional help, love and gratitude. Thank you all for believing in me and being there every time I needed you guys, even when physically far away.

I take the privilege to thank all who have helped me directly or indirectly during my study at IVRI.

Above all I thank God almighty for showering of his divine prudence throughout the journey of my life and also for giving me patience and strength to overcome the difficulties, which I crossed my way in accomplishment of this endeavor.

Date : 15.05.10
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Abbreviations

µg	:	Microgram
µl	:	Microlitre
µm	:	Micro metre
BLAST	:	Basic local alignment search tool
bp	:	base pair
CaPV	:	Capripox virus
CMLV	:	Camelpox virus
CPE	:	Cytopathic Effect
CPXV	:	Cowpox virus
DNA	:	deoxyribonucleic acid
dNTP	:	deoxynucleotide triphosphate
DW	:	Distilled Water
EB	:	Elution buffer
EDTA	:	Ethylene diamine tetraacetic acid
EMEM	:	Eagles Minimum Essential medium
Fig	:	Figure
g	:	Gravitational force
gm	:	Gram
GTPV	:	Goatpox virus
hr(s)	:	Hours(s)
IPTG	:	Isopropyl- ß-D-thio-galactopyranoside
IVRI	:	Indian Veterinary Research Institute
LAH	:	Lactalbumin hydrolysate
LB	:	Luria-Bertani medium
mg	:	Milligram
min	:	Minute
ml	:	Milliliter
MM	:	Maintenance Medium
mM	:	Millimolar
moi	:	Multiplicity of infection

NBCS	:	New Born Calf Serum
NCBI	:	National Center for Biotechnology Information
nt	:	Nucleotide
OIE	:	Office International des Epizooties
ORF	:	Open reading frame
PBS	:	Phosphate buffered saline
PCR	:	Polymerase chain reaction
pmol	:	picomole
QPCR	:	Quantitative polymerase chain reaction
RM	:	Residual moisture
rpm	:	revolutions per minute
SPPV	:	Sheeppox virus
SNT	:	Serum neutralisation test
Ta	:	Annealing temparature
TAE	:	Tris acetate EDTA
TCID 50	:	Tissue culture infective dose 50
TCRP	:	Tissue culture rinderpest vaccine
TE	:	Tris EDTA
UV	:	Ultraviolet
VACV	:	Vaccinia virus
VARV	:	Variola virus
v/v	:	Volume by volume
w/v	:	Weight by volume
WHO	:	World Health Organization
X- Gal	:	5-bromo-4-chloro-3- indolyl- ß -D- galactoside

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Chapter I

Introduction

Camel (*Camelus dromedarius*) is an important animal of the desert ecosystem, and is raised for milk, meat, fibre, hide and transport. The camel's optimal adaptation to the extreme climate permits its blooming in the world's hottest zones, but also imparts natural resistance to certain diseases. One of the most common important infectious and contagious diseases diagnosed in the Old-World camelids is camelpox. Considerable economic importance of camel pox is due to the high morbidity, a relatively high mortality in younger animals, loss of condition and weight in all ages, as well as a fall in milk production (Jezek *et al.,* 1983). The disease has been reported initially in Punjab and Rajaputana (India) during 1909 (Leese, 1909) and later from many other countries (Hafez *et al.,* 1992) including India (Chauhan and Kaushik, 1987; Marodam *et al.,* 2006). Camelpox occurs in almost every country in which camel husbandry is practised and outbreaks have been reported in the Middle East, Asia, Africa (Mayer and Czerny, 1990; Wernery *et al.,* 1997a, b) and in the southern parts of Russia and India. The disease is endemic in these countries and a pattern of sporadic outbreaks occurs with a rise in the seasonal incidence usually during the rainy season.

Camelpox is caused by camelpox virus (CMLV), which belongs to the family *Poxviridae,* subfamily *Chordopoxvirinae,* genus *Orthopoxvirus* (ICTV, 2005). CMLV is one of the least studied members of orthopox viruses. Direct contact or fomites are modes of transmission. The incubation period varies from 4 to 15 days with an initial rise in temperature followed by development of papules, vesicles, pustules and finally scabs. CMLV is host specific and does not infect other animals. It was earlier thought as a zoonotic agent (Leese, 1909; Davies *et al.,* 1975), but so far a little evidence has been documented (Kritz, 1982; Jezek *et al.,* 1983). The study of camelpox has been neglected because it presents no apparent risk to man or domestic animals in developed countries.

The presumptive diagnosis of camelpox infection can be based on clinical signs. Routinely, camelpox is diagnosed based on clinical signs, epizootiological and pathological findings, isolation of virus, electron microscopy, and genus-specific antigen capture ELISA (Czerny *et al.*, 1989) and by polymerase chain reaction.

Despite of its economic importance, little had been published especially on the vaccines till 1992. In earlier days, lactotherapy and heterologous vaccination methods have been followed to control camelpox. Nowadays, both live attenuated and inactivated vaccine are available in few countries. However, in India, so far attempts have not been made to develop camelpox vaccine. Furthermore, considering the disadvantages with inactivated vaccines viz. short duration of immunity and high antigenic mass required for vaccination of vast livestock population, live attenuated vaccine is the best choice as a long-term solution towards the control of camel pox. Hence, a live attenuated vaccine using local isolate of CMLV has recently been developed by the Indian Veterinary Research Institute (IVRI), Mukteswar and the vaccine is to be validated.

All vaccines lose potency over time and the rate of potency loss is temperature dependent. Furthermore, lyophilized vaccines are generally only stable prior reconstitution. Once they have been reconstituted, the potency of live-attenuated vaccines can drop rapidly. The vaccines in the cold-chain are sometimes accidentally subjected to temperatures both higher and lower than the target range. Hence, the vaccine formulations that are heat resistant to heat damage would have major benefits.

A range of excipients has been employed in the stabilizing dry vaccine formulations, and for optimal results, live viruses might require unique stabilizers that are able to protect membranes and internal molecules such as nucleic acids, as well as external structural proteins (Chen and Kristensen, 2009). All dry vaccine formulations currently in use require reconstitution with a liquid diluent prior to administration and hence, the appropriate diluent has to be used. The maximum temperature and durations that can be tolerated will need to be demonstrated. India being a subtropical country, it experiences wide range of temperatures. Therefore, evaluation of the vaccine for its 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.

Broadly, the efficacy (sero-conversion) and safety of live attenuated vaccine depends on the number of viable virus 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. To estimate the number of infectious viral particles in the live attenuated vaccine, commonly used assays include, $TCID_{50}$ or Plaque forming unit (PFU) in cell culture, or egg infective dose (EID_{50}) estimation in embryonated eggs respectively. However, these assays are time consuming, labor intensive, and 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), 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.

However, one drawback of QPCR, which relies on quantitation of virus nucleic acid is that, it will not differentiate between the live and dead viral particles. To overcome this drawback, cell cultures are infected with vaccine viruses and harvested at a particular (critical) time point and the QPCR will be employed with the DNA isolated from the harvest, as it is presumed that only the live viral particles can infect the cells. It is principally based on the fact that the quantity of the viral nucleic acid harvested at a particular time point is proportional to the amount of the viable virus used initially for cell culture propagation. QPCR would particularly be useful for the quality control of the camel pox vaccines, since vaccines are produced in Vero cells, in which virus titration is impractical and even the neutralization assay would be erroneous. As such, our attempt to use real-time PCR for quantitation of camelpox vaccine virus is a new approach and a novel development in the quality control of veterinary vaccines. The assay can be explored for its potential to replace or supplement the conventional end point dilution methods. Taking these aspects into account, the present work has been designed with the following objectives.

Objectives

(i) To estimate the potency of live attenuated camelpox vaccine by using real-time PCR

(ii) To evaluate the thermo-stability of the vaccine using different stabilizer combinations by end point dilution ($TCID_{50}$) method.

Chapter II

Review of Literature

2.1. Camelpox

Camel (*Camelus dromedarius*) is an important animal of the desert ecosystem. The camel population in India is mostly confined to Rajasthan, Haryana, Punjab, Gujarat, Madhya Pradesh and Uttar Pradesh (Marodam *et al.,* 2006). They are raised for milk, meat, fibre, hide and transport. As a result of frequent droughts and extending arid and semi-arid zones, there has been increased attention paid to camel health and welfare. The camel's optimal adaptation to the extreme climate of the desert opened new perspectives for the inhabitants of desert. The adaptability to the climatic extremes not only permits its blooming in the world's hottest zones, but also imparts natural resistance to certain diseases (Wernery, 1995a).

However, one of the most important infectious and contagious diseases diagnosed in the Old-World camelids is camelpox, which from an economic point of view, is possibly the most important orthopoxvirus disease (Azwai *et al.,* 1995). The disease has been reported initially in Punjab and Rajaputana (India) during 1909 (Leese, 1909) and later from many other countries (Hafez *et al.,* 1992) including India (Chauhan and Kaushik 1987; Marodam *et al.,* 2006). It occurs throughout the camel-breeding areas of Africa, north of the equator, the Middle East and Asia, where the camels are used as beast of burden and for milk and causing economic impact through loss of production and sometimes death. The study of camelpox has been neglected because it presents no apparent risk to man or domestic animals in developed countries, but camelpox is a regular cause of camel morbidity under the traditional nomadic management systems.

2.1.1. Etiological agent

Camelpox is caused by Camelpox virus (CMLV), which belongs to the genus *Orthopoxvirus* (OPXV) of subfamily *Chordopoxvirinae* and family *Poxviridae* (ICTV, 2005). The discovery of CMLV was a concern when it was described as smallpox-like disease during smallpox eradication campaign (Baxby, 1972). Both CMLV and VARV (Variola virus, the etiological agent of smallpox), cause illness in a single host species producing small, white pocks on the chorioallantoic membrane of a fertile hen's egg and grow at similar temperature and replicate in rabbit skin (Fenner *et al*., 1989). Despite these similarities, CMLV and VARV are distinguishable (Bedson, 1972; Baxby, 1974; Esposito and Knight, 1985) and CMLV has rarely, if ever, caused disease in man (Jezek *et al*., 1983). Likewise, VARV is unable to cause disease in camels, although camels immunized with VARV are resistant to subsequent infection with CMLV (Baxby *et al*., 1975). CMLV is one of the least studied members of orthopox viruses (Fenner *et al.,* 1989) till recently. CMLV is indistinguishable from the prototypic member of the family *Poxviridae*, Vaccinia virus (VACV) with respect to size, shape, structure, physico-chemical properties and replication (Gubser and Smith 2002; Sheikh Ali *et al.,* 2009). However, the genome structure and phylogenetic analysis of DNA sequences for all open reading frames (ORFs) indicate that CMLV is clearly distinct from VARV and Vaccinia virus (VACV). It has been suggested that VARV has been originated from a cowpox virus (CPXV)-like ancestor (Afonso *et al.,* 2002). The sequence of CMLV is most closely related to VARV (Gubser and Smith, 2002; 2007).

The average size of the virion is 224x389 nm (Borisovich, 1973). OPXVs are enveloped, brick-shaped and the outer membrane is covered with irregularly arranged tubular structures. Each virion consists of an envelope, outer membrane, two lateral bodies and a core. These are all a group of single molecule large double-stranded DNA viruses that replicate in the cytoplasm (Moss, 2001). The genome has cross-links that join the two DNA strands at both ends. The end of each DNA strand has long inverted tandemly repeated nucleotide sequences that form single-stranded loops and the central region of the genome contains genes that are highly conserved in all sequenced orthopoxviruses (Murphy *et al.,* 1999).

2.1.2. Epidemiology and geographical distribution

Although the causative virus was not isolated until 1970 (Sadykov, 1970), camelpox is known in almost all camel raising countries (Wilson *et al.,* 1990). Infections are commonly encountered in the herds of the nomadic pastoralists in the semi-desert zones (Egypt, Somalia, Kenya) of Africa (Tantawi *et al.,* 1974; Davies *et al.,* 1975, 1985; Kritz, 1982; Munz *et al.,* 1986a; Fassi-Fehri, 1987), the Middle East (Ramyar and Hessami, 1972; Al-Falluji *et al.,* 1979; Higgins *et al.,* 1992; Kaaden *et al.,* 1992; Al Hendi *et al.,* 1994), the former USSR (Sadykov, 1970; Marrenikova *et al.,* 1974) and some Asian countries (India, Pakistan, Afghanistan) (Leese, 1909; Chauhan and Kaushik, 1987). Camelpox occurs in almost every country in which camel husbandry is practiced apart from the introduced dromedary camel in Australia and tylopods (llama and related species) in South America (Hafez *et al*., 1992; OIE, 2008). During dry seasons, camelpox usually follows a milder course (Pfahler and Munz, 1989). However, epidemics are reported to occur in regular cycles which depend on the rainy season and relationship of the density of the insect population to the number of immune camels in the population.

The disease is endemic and outbreaks have been reported in the Middle East countries like Bahrain (Higgins *et al*., 1992), Iran (Baxby, 1972; Ramyar and Hessami, 1972), Iraq (Al Falluji *et al.,* 1979), Saudi Arabia (Hafez *et al.,* 1986, 1992; Al-Ani and Al-Salihy, 1988; Abu Elzein *et al*., 1999; Hussein and Al-Mufarrej, 1999; Ommer Dafalla and Abdelhamid Elfadil, 2007), United Arab Emirates (Kaaden *et al*., 1992; Pfeffer *et al*., 1996) and Yemen, in Asia [India (Leese, 1909; Chauhan *et al*., 1985, 1986; Chauhan and Kaushik, 1987; Khanna *et al.,* 1996), Afghanistan and Pakistan], in Africa (Renner- Muller *et al*., 1995) [Algeria, Egypt (Tantawi *et al*., 1974), Kenya (Davies *et al.,* 1975; Schwartz *et al.,* 1982; Gitao, 1997), Mauretania, Niger (Nguyen-ba-vy *et al.,* 1989), Somalia (Kriz, 1982) and Morocco, Ethiopia, Oman, Sudan (Fassi-Fehri, 1987; Shommein and Osman, 1987)] and in the Southern parts of Russia (Roslyakov, 1972; Buchnev *et al*., 1987). Recently, Al-Ziabi *et al*., (2007) has reported the first outbreak of camel pox in two provinces of Syria namely, Hama and Duma.

2.1.3. Nature, severity and impact of the camel pox infection

The occurrence of camelpox is associated with both tangible and intangible losses. The disease is accompanied by high morbidity, relatively high mortality in younger animals, weight loss and reduction in milk production and trade restriction of camels and their byproducts (Jezek *et al.,* 1983). The disease mostly affects young ones aged 2-3 years and outbreak in a herd is very often associated with weaning or poor nutrition. The morbidity rate of camelpox is variable and depends on the circulation of the virus in a herd. Serological surveys taken in several countries like Libya (Azwai *et al*., 1996), Kenya (Davies *et al*., 1985) and Sudan (Khalafalla *et al*., 1998) revealed a high prevalence of antibodies to camelpox (Wernery and Kaaden, 2002). Reported morbidity, mortality and case fatality rates (CFR) ranges between 30-90%, 1-15% and 25%, respectively (Al-Ziabi *et al.,* 2007). The incidence and CFR are mostly higher in males than females (Jezek *et al.,* 1983) and the mortality rate is greater in young animals than adults (Kritz, 1982). The mortality in adult animals ranged from 10 (Higgins *et al.,* 1992) to 28% (Jezek *et al.,* 1983) and in case of young animals it is 25 to 100% (Mayer and Czerny, 1990). Further, the mortality is influenced by the presence of inter-current diseases (notably trypanosomiasis), stress, the age, and the nutritional status of the animal and virus virulence (Davies *et al.,* 1975; Jezek *et al.,* 1983; Al Hendi *et al.,* 1994). Camelpox outbreaks are often temporal due to the movement of camels for grazing and watering, which results in mixing of the herds and the introduction of new camels into a herd (Azwai *et al.,* 1996). The role of insect in mechanical spreading of the infection needs to be investigated as most of other poxviruses are transmitted mechanically also. In most herds, the infection is frequently seen in the young camel crop as the acquired maternal immunity wanes after 5-8 months of birth (Nothelfer *et al.,* 1995). Clinically, camelpox occurs in the severe, generalized (seen mostly in young animals) and the milder, localized forms (in older camels).

2.1.4. Host range

In general, strains of camelpox virus (CMLV) have an extremely limited host range. Intra-dermal inoculation of the virus into sheep, goats, rabbits, guinea pigs, rats, hamsters, and mice have not been successful (Ramyar and Hessami, 1972; Bhanuprakash *et al.,* 2010). Apart from camels, the only animals that have been infected successfully are monkeys and infant mice (Baxby, 1972). Even in natural infection, sheep and cows which were in direct

contact with infected camels were healthy indicating that the CMLV is mostly host specific (Al-Zi'abi *et al.,* 2007). However, CP/Nw/92/2 isolates of CMLV in Sudan were able to produce local pox lesions in chickens also (Khalafalla and Mohamed, 1998).

2.1.5. Transmission

Transmission is by either direct contact between infected and susceptible animals or indirect infection via a contaminated environment (Al-Zi'abi *et al*., 2007). The infection usually occurs by inhalation or through skin abrasions. Virus is secreted in milk, saliva, and ocular and nasal discharges (Ramyar and Hessami, 1972). The dried scabs shed from the pox lesions may contain live virus for at least four months and potentially contaminate the environment (OIE, 2008). The role of an arthropod vector in the transmission of the disease has been suspected (Al-Ziabi *et al*., 2007) and the tick population during the rainy season is probably involved in the spread of the disease (Wernery *et al*., 1997a, b). However, other potential vectors such as biting flies and mosquitoes may also be involved.

2.1.6. Clinincal pathology

Clinical signs

The incubation period varies from 4 to 15 days with an initial rise in temperature followed by development of papules, vesicles, pustules and scabs. Clinical manifestation ranges from in-apparent and mild local infection (confined to the skin) to moderate and less common severe systemic infections, depending on the CMLV strains (Wernery and Kaaden, 2002). The disease is characterized by fever, lymphadenopathy and skin lesions (Wernery *et al.,* 1997a). Lesions appear 1–3 days after the onset of fever with erythematous macules and progress to the formation of papules, vesicles and pustules. Crusts may develop on the ruptured pustules. These lesions first appear on the head, eyelids, nostrils and the margins of the ears. In severe cases, the whole head may be swollen. In some of the acute cases, animals may show intense pruritis (Hussein and Al-Mufarrig, 1999). Later, skin lesions may extend to the neck, limbs, genitalia, mammary glands and perineum. In the generalized form, pox lesions may cover the entire body. Skin lesions may take up 4–6 weeks to heal. In the systemic form of the disease, pox lesions can be found on the mucous membranes of the mouth, respiratory and digestive tracts (Kritz, 1982; Wernery and Kaaden, 2002). In most cases, CMLV infection

was reported to be relatively benign, especially in adult camels. However, severe infections with a high CFR and blindness are common in young camels, while abortions, still birth, weight loss and reduced milk yield are unusually recorded in adult animals (Ramyar and Hessami, 1972; Higggins *et al*., 1992; Kritz, 1982; Khalafalla and Mohamed, 1996). The animals may show salivation, lacrimation and a mucopurulent nasal discharge. Diarrhoea and anorexia may occur in the systemic form of the disease. Death is usually due to septicaemia caused by secondary bacterial infections like *Staphylococus aureus* (Nothelfer *et al.*, 1995; Wernery and Kaaden, 2002).

Post Mortem Lesions

There are only a few reports about detailed pathological descriptions of internal camelpox lesions. The lesions observed on post-mortem examination of camels that die of severe camelpox infection are multiple pox-like lesions on the mucous membranes of the mouth, respiratory (mainly on trachea and lungs) and digestive tracts (Pfeffer *et al*., 1998a).The size of the lesions in the lungs may vary between 0.5 and 1.3 cm in diameter and occasionally may reach up to 4–5 cm in diameter. Smaller lesions may have foci of hemorrhagic centre on the surface of the lungs. In addition, infection of the heart and liver has also been observed in fatal form of the camelpox infection (Pfeffer *et al*., 1998a).

Histopathology

Histopathological examination reveals typical proliferative and vacuolar degenerative changes in the epithelial cells in affected areas. The early skin nodules show characteristic cytoplasmic swelling, vacuolation and ballooning of the keratinocytes particularly of the outer stratum spinosum. Rupture of these cells produces vesicles and localized oedema. Perivascular cuffing of mononuclear cells, neutrophils and eosinophils are often observed in the dermis. Marked epithelial hyperplasia may occur at the borders of the skin lesions (Yager *et al*., 1991). The lung lesions are characterized by hydropic degeneration, proliferation of bronchial epithelial cells and infiltration of the affected areas by macrophages, necrosis and fibrosis (Wernery *et al*., 1997a; Kinne *et al*., 1998; Pfeffer *et al*., 1998a). HE-stained lung sections reveal confluent foci of proliferative alveolitis and bronchiolitis in which the normal architecture is obliterated with necrosis and fibrosis. Immunohistochemical examination of these foci shows numerous poxvirus antigen positive cells in the bronchial epithelia (Kinne *et al*., 1998).

2.1.7. Immunity to infection or vaccination

Immunity against camelpox is dependant on both humoral and cell mediated immune response (OIE, 2008) though the circulating antibodies do not reflect the immune status of the animal (Wernery and Kaaden, 2002). Camelpox infections produce long lasting immunity in recovered animals. Live attenuated vaccine provides protection for at least 6 years (Wernery and Zachariah, 1999), whereas inactivated vaccine gives only one year of protection and demanding revaccination after 8 weeks (OIE, 2008).

2.1.8. Public health significance

The CMLV is host specific and does not infect other animal species, including cattle, sheep and goats. The CMLV was earlier thought as a zoonotic agent (Leese, 1909; Davies *et al.*, 1975) but so far a little evidence has been documented from Somalia in smallpox-unvaccinated individuals (Kriz, 1982; Jezek *et al.*, 1983). However, mild skin lesions in humans associated with camel pox have been reported (Coetzer, 2004), and accordingly only one suspected case of human camelpox has been described (Kritz, 1982), underlining that camelpox is of limited public health importance. Even surveys in camelpox enzootic areas strongly believe that camelpox is not transmissible to man. Although there have been previous reports in the literature that man can be infected through handling affected camels, experience during the smallpox eradication campaign in Somalia in 1978-1979 suggests that human camelpox very rarely, if ever, occurs (Jezek *et al.*, 1983). At times, the virus could be pathogenic for man as are cowpox and monkey pox (Marennikova *et al.*, 1974). It could be one of the potential bio-warfare agents (Balamurugan *et al.*, 2008). The probability of occurrence of CMLV infections for immunocompromised humans is higher (De Clercq, 2002). It is possible that clinical or subclinical manifestation of the camelpox infection in man could occur but no epidemiological evidence, primarily due to lack of well-described human cases. However, recently conclusive human cases of camelpox have been reported based on serology and molecular techniques in addition to clinical and epidemiological features (Bera *et al*., 2010). Immunological surveys for specific camelpox antibodies among unvaccinated herdsman at risk could, therefore, be helpful to determine the possible transmissibility of camelpox to man (Azwai *et al.*, 1996).

2.1.9. Diagnostic approaches

The presumptive diagnosis of camelpox infection can be made on the basis of clinical signs. However, infections of camels in the early clinical stages and in mild cases should be differentiated from contagious ecthyma (orf), which is caused by parapox virus, papilloma virus infections and insect bites. Several diagnostic methods are available for the diagnosis of camelpox (Pfeffer *et al.,* 1998b), but it is preferred to use more than one test for confirmatory diagnosis.

Parapoxviruses (PPV) and papillomaviruses have also been associated with skin infections of camel similar to camelpox lesions (Roslyakov, 1972; Kriz, 1982; Buchnev *et al.,* 1987; Renner-Muller *et al.,* 1995; Wernery and Kaaden, 1995b; Munz *et al.,* 1997). Therefore, these infections cannot be differentiated based on clinical signs. Routinely, camelpox is diagnosed based on clinical signs, epizootiological and pathological findings (Buchnev *et al.,* 1987), isolation of virus in cell lines, electron microscopy, and genus-specific antigen capture enzyme linked immunosorbent assay (Davies *et al.,* 1975; Czerny *et al.,* 1989; Mayr and Czerny, 1990; Johann and Czerny, 1993; Murphy *et al.,* 1999). Camelpox could be differentiated from other OPXV and parapox infections by the use of restriction enzyme analysis (REA) (Murphy *et al.,* 1999) and transmission electron microscopy (TEM) (Al-Zi'abi *et al.,* 2007). Recently, C18L gene based species specific PCRs (in conventional and real-time formats) have been developed to differentiate CMLV from BPXV, and other OPXVs (Balamurugan *et al.,* 2009).

(i) Transmission electron microscopy

Transmission electron microscopy (TEM) is a rapid method to demonstrate CMLV in scabs or tissue samples. The laboratory confirmation of camelpox is by the demonstration of the characteristic, brick-shaped orthopox virions in skin lesions, scabs (Al-Zi'abi *et al.,* 2007) or tissue samples. The virus is distinct from ovoid-shaped parapox virus, the aetiological agent of the camel contagious ecthyma (orf). Relatively high concentration of virus in the sample is required for positive diagnosis and it is not possible to differentiate CMLV from other OPXV species. However, currently, TEM is the best method for distinguishing clinical cases of camelpox and orf caused by camelpox and parapox viruses respectively, although the viruses can be differentiated by serological and PCR assays (Mayer and Czerny, 1990).

(ii) Immunohistochemistry

Camelpox can be confirmed by immunohistochemical demonstration of the camelpox antigen (OPXV fusion protein) in scabs and pox lesions in tissue (Nothelfer *et al.,* 1995). It is a fast method and can be used in lieu of TEM to establish a tentative diagnosis (Wernery and Kaaden 2002). In addition, the paraffin-embedded samples can be stored for a long period, enabling future epidemiological and retrospective studies. Both monoclonal and polyclonal antibodies can be used. However, almost any polyclonal antibody against VACV is likely to produce results in this test, as there is a wide homology between VACV and CMLV (Nothelfer *et al.,* 1995).

(iii) Virus isolation

Camelpox virus was first isolated in 1969 (Buchnev and Sadykov, 1969). Since then, many countries have reported the virus isolation (Ramyar and Hessami, 1972; Baxby, 1974; Marennikova *et al.,* 1974; Tantawi et al., 1974; 1978; Davies *et al*., 1975; Al-Falluji *et al.,* 1979; Chauhan and Kaushik, 1987; El-Kenawy *et al.,* 1989; Nguyen-ba-vy *et al.,* 1989; Bhanuprakash *et al*., 2010). The most useful systems that can be considered for CMLV isolation are described below;

Embryonated chicken eggs

CMLV can be isolated on the chorioallantoic membrane (CAM) of 11 to 13-day old embryonated chicken eggs (ECE). The eggs should be incubated at 37°C and after 5 days, characteristic dense, greyish white pock lesions can be observed on the CAM. CMLV does not cause death of inoculated ECE. The optimum temperature for the formation of pock lesions is 38.5°C. If the eggs are incubated at 34.5°C the pocks are flatter and a haemorrhagic centre may develop (Tantawi *et al.,* 1974). Opaque white proliferative pock lesions of about 0.5 – 0.6 mm diameter were demonstrated on the CAM on 5th day without any haemorrhagic lesions but with stunted growth (Chauhan and Kaushik, 1987; Marodam *et al*., 2006). Characteristic long, opaque, white proliferative – tigroid – pock lesion has been produced when Vero cell adapted virus was inoculated onto CAM (Marodam *et al*., 2006).

Cell cultures

Various cell lines are susceptible to CMLV and some of them are HeLa, GMK-AH1, BSC-1, WISH and Vero (Baxby, 1972). CMLV can also be propagated in MA- 104 and MS monkey kidney, and baby hamster kidney (BHK) cells. Primary cell cultures like lamb testis, lamb kidney, camel embryonic kidney, calf kidney, and chicken embryo fibroblast (Tantawi *et al.,* 1974; Davies *et al.,* 1975) can also be used. CMLV shows typical cytopathic effect on a wide variety of cell cultures. Intracytoplasmic eosinophilic inclusion bodies, characteristic of poxvirus infection, may be demonstrated in infected cells using haematoxylin and eosin staining. The characteristic CPE includes rounding, vacuolization, multinucleated giant cell formation, syncytia and cytolytic changes in Vero cells (Pfeffer *et al*., 1996; Marodam *et al*., 2006).

(iv) Serological tests

Poxvirus antibodies can be detected in animal sera much more frequently than their isolation (Marrennikova, 1975). All viruses in the genus *Orthopoxvirus* are serologically cross-reactive. Parapox and CMLV do not cross-react and so infections of camelpox and camel orf can be distinguished serologically. Most of the conventional serological tests are time consuming and labor intensive. However, these could be used as adjunct for confirmation and retrospective epidemiological studies in those areas where vaccination against camelpox is not practiced. A wide range of serological tests are available to identify camelpox. Conventional serological tests like haemagglutination (HA), haemagglutination inhibition(HI), neutralization, complement fixation (CFT) and fluorescent antibody tests have been used to detect CMLV antibodies (Marrenikova *et al.,* 1974; Davies *et al.,* 1975; Schgal, 1977; Tantawi *et al.,* 1978; Al-Falluji *et al.,* 1979; Al Hendi *et al.,* 1994).

Serum neutralization test

Neutralization is the most common and confirmatory test for diagnosis of OPXV infections (Boulther *et al.,* 1971). Commonly, the test sera are titrated against a constant CMLV (100 $TCID_{50}$) (OIE, 2008).

Fluorescent antibody test

Fluorescent antibody test will also determine the status of antibodies in camels infected with CMLV. Although, there has been cross-fluorescence between virus and antisera of members of the Orthopox virus groups (including H 520 CMLV, VACV and CPXV), other related viruses such as capripox, avipox and parapox viruses shows no cross-fluorescence on indirect fluorescent antibody test (IFA) (Davies *et al*., 1975).

Enzyme-linked immunosorbent assay (ELISA)

ELISA and Western blotting have been developed recently (Azwai *et al.,* 1996), for the detection of IgG and IgM antibodies to CMLV in camel sera and OPXVs can be differentiated from parapoxvirus infections in camels. As ELISA is simple, it can successfully be applied for retrospective and also for epidemiological investigations. The test is more sensitive than VNT, HI, PHA and IFA tests for detecting antibodies to VACV and MPXV viruses (Marennikova *et al*., 1981). Further, VNT used for OPXV infection detection do not measure antibodies to envelope antigens, which are the prime agents in CMLV pathogenesis (Boulther *et al.,* 1971). In such instances, specificity of a test is of prime importance to differentiate OPXVs and PPVs, which are known to cause similar pox-like lesions in camels (Jezek *et al.,* 1983; Munz *et al.,* 1986b; 1990). Likewise, 95% prevalence of camel pox in camels from Kenya, Somalia and Sudan has been reported using ELISA (Munz *et al.,* 1986a; Pfahler *et al*., 1986), in which semi-purified CMLV antigen was employed. Later, in order to increase the specificity of ELISA for CMLV, a purified CMLV was used in ELISA (Azwai *et al.,* 1996), which resulted in low background signal with the negative control camel sera. Therefore, like in other diseases use of purified antigen in CMLV-ELISA can be employed.

Western blot analysis

One step ahead of ELISA, Western blotting is applied for the confirmation of the specificity of the ELISA. When, blotting pattern of MPXV, VACV, BPXV, CPXV and ectromelia viruses, (Arita and Tagaya, 1977; Esposito *et al*., 1977; November *et al*., 1989) was compared, three characteristic bands of MW 23, 31 and 35 kDa for CMLV were obtained and which enabled to differentiate CMLV from the above listed OPXVs. CMLV is identified from the VACV group by the absence of a 23 kDa protein band and is distinguished from all

others by possessing 31 and 35 kDa protein bands. Although the 23 kDa CMLV band has been found in other OPXVs, it is absent in MPXV (human and monkey isolates). 31 and 35 kDa proteins are present in all CMLV positive camel sera (Azwai *et al.,* 1996). If EM facility is not available, ELISA followed by Western blotting is useful in differentiating CMLV from other OPXVs.

(v) Polymerase chain reaction

The presence of viral nucleic acid may be confirmed by polymerase chain reaction, and different strains of CMLV may be identified using DNA restriction enzyme analysis. The PCR is a fast and sensitive method for the detection of OPXV DNA. EM is the fastest technique for poxvirus diagnosis, but has poor sensitivity, and the equipment is not always available. Virus isolation and identification is a sensitive method, but it is not a rapid method. Virus isolation in cell culture detects only live virus particles, and therefore particles that have been inactivated during processing of scabs or transportation to the laboratory may not be detected as pointed out by Sullivan *et al*. (1994) that many scab specimens are PCR positive but virus isolation negative.

Genus (Orthopoxvirus) specific PCR

A generic PCR assay developed by Meyer *et al.* (1994), allows the detection and differentiation of *Orthopoxvirus* species because of the size differences of the amplicons based on the gene sequence encoding the A-type inclusion protein (ATIP). This method yields 881 bp CMLV specific amplicon whereas, hemagglutinin (HA) – gene specific PCR yields a product of 1100 bp (Damaso *et al*., 2000). The amplification of genes could be achieved by using template DNA derived from homogenized skin biopsy and from infected Vero tissue cultures (Al-Zi'abi *et al.,* 2007; Balamurugan *et al.,* 2009).

Species- specific (Camelpox virus) single PCR

Tumor Necrosis factor-binding protein receptor-II (TNFR-II) gene based PCR was used as a quick diagnostic tool to confirm the CMLV infection. An amplicon of 270 bp specific to CMLV can be amplified (Lapa *et al*., 2002; Marodam *et al*., 2006). Recently, a PCR assay based on the C18L gene (encoding ankyrin repeat protein) has been developed, which yields a specific amplicon of 243 bp of the C18L gene in CMLV suspected cases

(Balamurugan *et al.*, 2009) with sensitivity limit of 0.4 ng of viral DNA. This method is highly useful for the direct detection and differentiation of CMLV from other OPXVs, capripoxviruses (CaPVs), and parapoxviruses in both cell culture and clinical materials. This PCR method has been succefully adapted to SYBR Green dye based real-time formats (Balamurugan *et al.*, 2009). The primers for this PCR were designed based on published C18L gene sequences (Accession # AY243312, EF205289, EF 205282-83).

Duplex PCR

Diagnostic PCR based on highly conserved regions of a particular gene(s) of a virus can be of immense value for differential diagnosis. Therefore, for routine diagnosis of a virus of diverse origin, PCR using specific primers designed on the basis of two different genes to avoid false-negative results are highly preferable and DNA pol gene is the most conserved nonstructural protein among the viruses in OPXV genus and is group specific (Sahay, 2006). In view of this, Balamurugan *et al,* (2009) developed a duplex PCR based on the C18L and DNA polymerase (DNA pol) genes for specific and rapid detection and differentiation of CMLV from BPXV. This is a much more appropriate assay than an assay solely based on OPXV-specific ATI or HA genes (Al-Zi'abi *et al.*, 2007) for the simultaneous detection and differentiation of CMLV. The standardized duplex PCR, using a specific set of primers in a single reaction, yields both DNA pol (96 bp) and C18L (243 bp) gene products in CMLV samples, and only a 96-bp amplicon in other OPXVs, such as BPXV. The analytical sensitivity of this duplex PCR has limitations due to the different sensitivity that is attained for either the C18L or DNA pol gene-based PCR.

Further, Khalafalla *et al.* (2003) reported a PCR for diagnosis and differentiation of OPXV and PPV infections in camel based on the sequences of major envelope gene (B2L) of Orf virus NZ 2. Likewise, a PCR based on HA gene of CMLV has been reported to differentiate between OPXV species including CMLV (Ropp *et al.*, 1995) and this assay was successfully used to identify CMLV DNA in clinical materials, infected cell culture and CAMs.

Fluorescence resonance energy transfer real- time PCR assay

Based on the variability of the A36R gene (Huemer *et al,*. 2008), Panning *et al.* (2004) used fluorescence resonance energy transfer real-time PCR assay (FRET) for the

detection, differentiation, and quantification of VARV, MPXV, CPXV, CMLV and VACV; the method has advantages like rapid turnaround time, relative freedom from cross-contamination free, ability to differentiate strain, and the provision to use multiplexed probes. Detection of OPXV DNA by real-time PCR was used by Nitsche *et al*. (2004) who have concluded that the assay was rapid and reliable for OPXV diagnosis and VARV. C18L gene based real-time PCR using SYBR Green chemistry for detection of CMLV DNA from scab samples has also been reported (Balamurugan *et al*., 2009).

(vi) Restriction enzyme profiling

Restriction enzyme (RE) analysis of viral genomic DNA allows comparison of CMLV isolates. The information on the genome characterization of CMLV is very scanty, though the virus is closest to VARV (Afonso *et al.*, 2002; Gubser and Smith, 2002; Gubser *et al.*, 2007). Examination of the genome organization of CMLV has revealed a distinct pattern after *Hind III* digestion (Fenner *et al.*, 1989). Comparative RE analyses of genomic DNA of five isolates of camels from different geographical regions of Africa and Asia using *Hind III* and *Xho I* demonstrated minor differences between them (Renner-Muller *et al.*, 1995; Pfeffer *et al.*, 1996), which is similar to the conclusions drawn for CMLV isolates from Dubai but was contrary to RE profile of VACV strains (Pfeffer *et al.*, 1996). However, no major differences from the vaccine strain have so far been demonstrated (Wernery *et al*., 1997b).

(vii) Sequencing and phylogenetic analysis

Comparative sequence analysis of the B5R major envelope protein (Engelstad and Smith, 1993; Isaacs *et al.*, 1992; Martinez-Pomares *et al.*, 1993) of the Indian CMLV isolates revealed 95.8 to 96.2%, homology with those of VARVs at amino acid (aa) levels, however, the homology with other OPXVs was ranged from 90.3 to 94.7%. Similarly, comparative analysis of the C18L gene (encodes ankyrin repeat protein) sequences of Indian CMLV isolates with other OPXVs revealed that the targeted gene was relatively divergent and CMLV isolates have 99.3–99.6% and 98.7–99.3% identity among themselves at the nucleotide (nt) and aa levels, respectively (Balamurugan *et al*., 2009). But, the sequence identity of CMLV with BPXV was only 59–59.3%, whereas variable identity was noticed with other OPXVs (92.1– 96.2%). At aa level, CMLV showed a sequence identity of 35.4–36.8% with BPXV

and 85.3–93.4% with other OPXVs. However, an unique change of aa G147D was noticed in the Indian CMLV isolates and hence, CMLV sequences cluster separately in a group and differ from other OPXV members in a phylogenetic tree.

2.1.10. Therapeutic management

Post exposure treatment of camelpox infection has not yet been described. Treatment of severe cases includes minimization of secondary infections by topical applications or parenteral administration of broad-spectrum antibiotics and vitamins (Wernery and Kaaden, 2002). The alternative treatment could be use of antiviral agents such as cidofovir which is useful to manage camelpox infections, particularly in young camels. In addition to cidofovir, several acyclic nucleoside phosphonates (ANPs) including HPMP-5-azaC, cHPMP-5-azaC, and HDE-cHPMP-5-azaC, represents promising candidates for treating poxvirus infections (Smee *et al.,* 2002; Duraffour *et al.,* 2007).

2.1.11. Prevention and Control

Despite of its economic importance, little had been published especially on the production of vaccines till 1992. Earlier reports on camelpox vaccine were first published from the Soviet Union (Samartsev and Praksein, 1950; Buchnev and Sadykov, 1967; Borisovich, 1973; Sedov, 1974), however the details in respect of strain, safety and effectiveness were not available. In earlier days, a primitive method viz. lacto-therapy (scarification of a mixture of milk and camelpox infected crusts) had been followed in Punjab (Lesse, 1909), Russia (Krupenko, 1973) and Bedouin (Higgins, 1986) to control camelpox. Later only in 1973, there were reports of availability of camelpox vaccines from Russia, which were made from VACV rather than CMLV (Buchnev *et al.,* 1987). Because of close antigenic relationship between CMLV and VACV, it was possible to immunize camels with vaccinia strains. Therefore, two camelpox outbreaks in Bahrain ((Mayr *et al.,* 1984; Higgins *et al*., 1992) and Iran (Baxby *et al*., 1975) were successfully controlled, when camels were vaccinated with vaccinia- Elstree strain/Lister strain and vaccinia-strain EA8, respectively.

Later, various reports of inactivated camelpox vaccines from different countries (Buchnev and Sadykov, 1967; Mayr, 1999) with variable success were reported. Similarly, several reports of inactivated camelpox vaccines from Morocco (EL-Harrak *et al*., 1991;

1998), Saudi Arabia (Hafez *et al*., 1992) and the UAE (Kaaden *et al*., 1992, Wernery and Kaaden, 1995b; Wernery *et al*., 1997a; 2000; Wernery and Zachariah, 1999) have been reported later. Inactivated vaccine provides only 12 month protection and a large dose of virus is required to elicit protective antibody level.

In the due course, attenuated virus strains were employed in Saudi Arabia and in the UAE for the production of live attenuated vaccine. The UAE group established a permanent fetal dromedary skin cell line (Dubca) for the isolation of the CMLV (Kaaden *et al*., 1992; Klopries, 1993; Kaaden *et al*., 1995). The UAE attenuated CMLV vaccine (called Ducapox = Dubai camelpox vaccine) has been used since 1994 with a great success. Ducapox® is commercially produced in South Africa. It also protects new world camelids against camelpox (Wernery *et al*., 2000). A maximum of six years protection has been reported in a year old camel after vaccination with "Ducapox®" in United Arab Emirates (Wernery and Zachariah, 1999). However, the authors have stressed that only a small number of camels were used in this long term experiment and recommended a booster in 6-9 month old camels to avoid any vaccine breakdown because of maternal antibody. This was a live attenuated vaccine produced by Onderstepoort Biological Products (OBP), Republic of South Africa (RSA), but the virus was originally isolated in United Arab Emirates and attenuated through serial passage in Vero cells. In UAE, both inactivated and live attenuated vaccines have experimentally been tried. Subsequently, a Saudi isolate of CMLV, propagated on monolayers of camel kidney cell cultures (CKCC) up to 78th passage was found safe and potent (10^3 to 10^4 $TCID_{50}$ / dose) in camels. The vaccine virus was designated as Jouf-78 and successfully employed for the production of live attenuated cell culture camelpox vaccine (Hafez *et al.*, 1992) and commercially manufactured as "Orthovac®" manufactured by Jordanian Vaccine Company (JOVAC) is currently used in many countries viz. Afghanistan, Albania, Bahrain, Ethiopia, Iraq, Jordan, Kuwait, Lebanan, Libya, Malaysia, Oman, Pakistan, Syria, United Arab Emirates, and Yemen. A similar vaccine is also available in Egypt, Morocco and Russia (apps.cfsph.iastate.edu). Both, "Ducapox®" and inactivated camelpox vaccines were found safe in pregnant camels (Khalafalla and EI-Dirdiri, 2003).

However in India, despite of economic importance and occurrence of sporadic cases of camelpox, so far attempts have not been made to produce camelpox vaccine. Furthermore,

considering the disadvantages with inactivated vaccines viz. short duration of immunity and high antigenic mass required for vaccination of vast livestock population, a live vaccine is the best choice as a long-term solution towards control of camel pox. Hence, a live attenuated vaccine using local isolates of CMLV has recently been developed by the IVRI and the vaccine is to be field validated.

2.2. Thermo-stability of live attenuated vaccines

The stability of a viral vaccine depends on several factors viz., heat sensitivity between viral types, the presence and nature of a stabilizer, the pH value of the vaccine, and the vaccine container. This is a serious drawback to the efficient use of the live attenuated vaccine in the enzootic areas like India with tropical/subtropical climate. In addition, these regions are usually having poor infrastructures and under these conditions it is difficult to maintain a cold-chain to ensure the efficacy of the vaccine. Further, it is necessary that each animal to be vaccinated should receive a minimum dose recommended by OIE i.e. 10^3 $TCID_{50}$. To maintain minimum immunizing dose in the field condition, maintenance of cold-chain is essential which requires highest recurrent cost. Therefore, to determine the optimal conditions for storage and its use in the field level, information on the stability of the vaccine is of great importance.

All vaccines lose potency over time and the rate of loss is temperature dependent. Therefore, cold-chain systems have been established to ensure that the potency of vaccines is maintained by storing them under refrigerated conditions until the point of use. For majority of existing, commonly used vaccines, a shelf life of 2 years or longer at 2-8°C is possible (Chen and Kristensen, 2009). However, the sensitivity of vaccines to excursions outside this range varies widely. Furthermore, lyophilized vaccines are generally only stable prior reconstitution. Once they have been reconstituted, the potency of live-attenuated vaccines can drop rapidly.

Experience has shown that it is possible to establish a cold chain for vaccine storage and distribution in any country. However, the effort is a major undertaking, maintenance is difficult and the capacity of the existing systems is under increasing pressure as new vaccines are developed. It is also apparent that, despite best effort, cold chains do not always function as intended for many reasons, such as improperly maintained or outdated refrigeration equipment, loss of power or lack of fuel to operate equipment, poor compliance with cold-

chain procedures, inadequate monitoring and poor understanding of the dangers of vaccine freezing (Wirkas *et al.,* 2007). The result is that vaccines in the cold-chain are sometimes accidentally subjected to temperatures both higher and lower than the target range.

Excessive heat can damage vaccines in a number of ways altering the tertiary structure of proteins and reducing the infectivity of the vaccines (Chen and Kristensen, 2009). The heat damage to a vaccine could be the direct result of inadvertent exposure to elevated temperature or, in the case of lyophilized vaccines, heat shock from the addition of diluent that is too warm. Heat damage is cumulative: the higher the temperature and longer the duration of exposure, the more sensitive the degradation.

Hence, the vaccine formulations that are heat resistant to heat damage would have major benefits, including reducing vaccine wastage, helping to ensure the effectiveness of vaccines, being less dependent on cold-chain supplies and equipment, extending the outreach of immunization programs by facilitating outside the cold chain (OCC) delivery of some vaccines and enabling vaccination activities to continue in emergency situations (eg: earthquakes or tsunamis) when the cold chain might well break down (Levin *et al.,* 2007).

Liquid formulations of vaccines have been the 'default' presentation since the introduction of vaccines. Freeze-dried formulations were only produced if necessary to achieve adequate stability. Some vaccines, notably many live attenuated vaccines, are particulary unstable unless stored as a dry product with a low residual moisture content (typically <3%) (Burke *et al.,* 1999). Lyophilized vaccines are freeze resistant and reasonably stable if stored in the cold chain. Once the dried product is reconstituted, potency often decreases rapidly, especially if the product is not kept cold (WHO, 2006).

A range of excipients has been employed in the stabilizing dry vaccine formulations, but some general features apply to many of the approaches. Non reducing sugars, such as trehalose or sucrose, with relatively high glass-transition temperatures are typically used as the primary excipients (Chen and Kristensen, 2009). Upon drying, these sugars form glass as opposed to crystals to maximize the stabilization effects. Trehalose and sucrose have been shown to be very effective stabilizers and are believed to act by hydrogen bonding to the dried protein and acting as a water substitute (Kaushik and Bhat, 2003). It has been proposed that

the amorphous character of the glass enables the intimate contact required for the formulation of hydrogen bonds to occur between sugar and protein (Cicerone and Soles, 2004).

Considerable success has been obtained using stabilizing inactivated protein and protein-polysaccharide conjugate vaccines. However, developing thermostable dry formulations of live attenuated vaccines has proved to be problematic, presumably owing to their more complex structures and the requirement of maintaining their viability. It is very likely that the composition of stabilizing formulations and the drying parameters used will need to be customized for each vaccine. For optimal results, live viruses might require unique stabilizers that are able to protect membranes and internal molecules such as nucleic acids, as well as external structural proteins (Chen and Kristensen, 2009).

All dry vaccine formulations currently in use require reconstitution with a liquid diluent prior to administration. While reconstitution might appear to be a straightforward process, in practice it is known to be a potential source of errors: the correct volume is not always matched to the vaccine, incorrect volumes of diluents might be used, reconstitution might not be undertaken in sterile conditions or multidose vials can become contaminated as doses are withdrawn from the vial.

Thermostable formulations that permit OCC storage of vaccines for part of their distribution will result in periodic temperature swings or excursions. The maximum temperature and durations that can be tolerated will need to be demonstrated. Reducing dependent on the cold chain by developing thermostable vaccine could have great economic and health benefits by reducing vaccine wastage and preventing the health consequences of administering damaged, ineffective vaccines to the patients (Chen and Kristensen, 2009).

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. 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) and it was seen that compared to the vaccine virus, thermo-resistant virus multiplied at higher temperatures (40^0C). On evaluation of both viruses, immunogenic protein profiles were found 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 56^0C. 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 (Mariner *et al.*, 1990b). Recently, the Indian Veterinary Research Institute (IVRI), Mukteswar, have been successful in developing intrinsic live attenuated Tr-PPR vaccines (goat and sheep isolates) employing thermo-stable Vero cell line which is adapted to grow at 40^0C. 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 (unpublished laboratory data). In an another study conducted by our laboratory, thermostability of high passaged (P100) GTP vaccine was carried out using LAH + 10% Sucrose (LS), 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, 37, 25 and 4°C and titrated. LHT found relatively superior than LHS and T-10; Relative difference of stabilization between LS 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 and 98 days at 4°C was observed (Unpublished laboratory data).

2.2.1. Chemical stabilizers for vaccine

Materials which are usually used as stabilizers for vaccines include amino acids, (arginine, cysteine, histidine, lysine, sodium glutamate), monosaccharides (glucose, galactose, fructose, and mannose), disaccharides (sucrose, maltose, and lactose), sugar alcohols (sorbitol and mannitol), polysaccharides (oligosaccharide, starch, cellulose, and derivatives), human and bovine serum albumin and gelatin, and gelatin derivatives (hydrolyzed gelatin, divalent cations and ascorbic acid as an antioxidant). However, the stabilizing effects of these materials are generally poor when they are used individually. Therefore, these stabilizers are usually employed in combination. For example, amino acids, saccharides, sugar alcohols and peptones can be used in combination, so that the stabilizing effect can be synergistically or additively enhanced.

Numerous studies on the thermo-stability of Rinderpest (RP) vaccine have been performed using bovine kidney cells and lyophilized with a variety of chemical stabilizers. It has been reported that defibrinated or citrated infected blood (Cheng and Fischman, 1949; Scott, 1954) and peptone water (Nguyen–Ba Luong *et al.,* 1959) can increase the half-life of attenuated RPV. A combination of 5% LAH and 10% sucrose has been a superior additive (Plowright *et al.,* 1970). A comparative study of various chemical stabilizers using three different formulations of stabilizers viz., 5% LAH and 10% sucrose (LS); lactobionic acid, hydrolyzed gelatin and sorbitol; buffered hydrolyzed gelatin and sorbitol (BUGS) has been carried out (Mariner *et al.,* 1990a). In this study, BUGS was found superior additive for the virus with less titer, whereas, LS for the virus with high harvest titer.

Of late, heavy water (D_2O) has also been exploited as stabilizer not only for viruses but also for cells and other biological macromolecules. D_2O has been assessed for its stabilization effect on poliovirus vaccine (Wu *et al.,* 1995) and yellow fever vaccine (Wu *et al.,* 1995; Adebayo *et al.,* 1998). Studies on the growth of poliovirus in media containing D_2O (Fiszman *et al.,* 1970) demonstrated that D_2O could modify viral yield and influence virion development at optimal and supra-optimal temperatures.

Economical and simpler process of production of thermo-stable live attenuated RP and PPR vaccine has been reported using trehalose dihydrate as stabilizer (Worral *et al.,*

2001). It has been found that PPR virus requires higher levels of trehalose than that of RP virus to attain same degree of thermo-tolerance. Recently, Sarkar *et al.* (2003) have also reported the thermo-tolerance of an indigenous live attenuated PPR vaccine (Sungri strain 1996) in India. The study inferred that the PPR vaccine lyophilized with either LS or Trehalose dihydrate is more stable and have a shelf life of at least 45 days at 4°C, 15-19 days at 25°C and 1-2 days at 37°C. At 45°C, BUGS had a marginal superiority and lasted for few hrs. It was further concluded that the live attenuated vaccine stabilized with LS, Trehalose dehydrate and BUGS require cold-chain during transit at ambient temperatures.

Riyesh (2008), reported that the PPR vaccine freeze-dried with LS will have an expiry period of 22 days at 25°C, 6-7 days at 37°C, 3-4 days at 40°C, 40-42 hours at 42°C and 23-27 hours at 45°C. However on the basis of half-life, a stabilizer with trehalose dihydrate, amino acids and divalent cations appeared better. The study showed that the vaccine could be transported at ambient temperatures (37°C) without the need of cold-chain for 6-7 days. Additionally, vaccine in reconstituted form appears safe to use up to 24 hrs post dilution at ambient temperatures, which is normally observed under field conditions especially countries like India.

In this regard, the present study was carried out on the evaluation of the thermo stability of the developed CMLV vaccine; thereby, once the suitable extrinsic stabilizer have been identified the vaccine can be transported across the country without the requirement of cold-chain and efficiently be used for vaccination program with minimal loss of potency at ambient temperatures.

2.3. Potency estimation of live attenuated Vaccines

Vaccines, whether developed conventionally or constructed by biotechnological means, must be evaluated for efficacy and safety before being released to the field. The potency of modified live virus (MLV) vaccines can be determined *in-vivo* in either the target host or in a suitable laboratory animal model, or *in vitro*. *In vivo* determination may be based on assaying the relation between dose and protection, a titration of the virus, or on the serological response induced in either the target species or a laboratory animal model. To reduce the use of laboratory animals, efforts should be made to replace *in vivo* titration of vaccine virus by titration in a suitable cell culture system.

Unlike inactivated vaccines, attenuated live vaccines (ALV) require an *in vivo* potency assay on each batch, the whole dose–response curve of MLV 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 virus (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 MLV 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 ($TCID_{50}$) estimation were used as standard assays for estimation of the infectivity of the MLV. Even though the plaque assay is considered to be the standard assay for estimation of number of infectious viral particles, the 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 (Umino, 1995; Fukuda *et al.,* 1996). 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).

These assays even though used from decades as the classical methods to estimate infectious particles, they have certain drawbacks. They are time consuming, labor intensive and prone to variation as the results mainly depend on subjective interpretation. Further these assays are not suitable for estimation of number of infectious viral particles in combined or multivalent live vaccines, as the CPE produced by each virus in the cell culture system is indistinguishable. Hence development of novel method, which is quicker, sensitive and specific, is imperative. Such methods could be potentially used in concert or to replace the existing methods of potency assays for live vial vaccines.

2.3.1. Novel potency assays - QPCR for MLV

Quantitative polymerase chain reaction (QPCR) has been frequently employed in general virological studies particularly, in the detection and quantitation of viruses. It offers significant improvements in the estimation of the viral load with high sensitivity with an ability to detect at least 10 log copies of the templates (Plumet and Gerlier, 2005). Quantitation of the viral load is valuable in assessing the degree of active infection, antiviral response studies, virus-host cell interactions and monitoring the disease progression as viral load is directly proportional to the prognosis of a disease.

SYBR Green and TaqMan probes are the most commonly used QPCR chemistries, although, a number of new formats are now available. The main principle behind SYBR Green dye based QPCR is that the dye binds to double stranded DNA and undergoes conformational change that increases the fluorescence activity of SYBR Green dye (Edwards *et al.,* 2004). The TaqMan probe based QPCR works on the principle of detection of a fluorescent signal produced proportionally during the amplification of a PCR product. A probe (TaqMan) is designed to anneal to the target sequence between the traditional forward and reverse primers. The probe is labeled at the 5' end with a reporter fluorochrome and a quencher fluorochrome added at any T position or at the 3' end. As long as both fluorochromes are on the probe, the quencher molecule stops all fluorescence by the reporter. However, as *Taq* DNA polymerase extends the primer, the intrinsic 5' to 3' nuclease activity of *Taq* polymerase degrades the probe, releasing the reporter fluorochrome. The amount of fluorescence released during the amplification cycle is proportional to the amount of product generated in each cycle.

Over recent years, QPCR has been used as novel method for the potency estimation of live vaccines particularly the multivalent vaccines to overcome the problems of cell culture methods of virus titration. Schalk *et al.* (2004) reported QPCR to estimate the number of infectious measles viral particles in combined live attenuated mumps-measles-rubella (MMR) vaccine. The same methodology has been employed to assign the infectious potencies to adenovirus-based vaccines and vectors for gene therapy (Wang *et al.,* 2005) and to determine *in vitro* infectious particles of pentavalent reassortant rotavirus vaccine (Ranheim *et al.,* 2006). In these experiments, cells were infected with serially diluted test sample and a reference vaccine and assay controls followed by incubation of all the samples. After certain time, the

cells were harvested; nucleic acid extracted and quantified using QPCR. Since specific primers and probes were used, potency of each component of the pentavalant vaccine can be estimated in the presence of other components, and results were comparable with classical assays (Plaque assay and $TCID_{50}$).

2.3.2. QPCR for the detection and quantitation of poxviruses

A number of conventional PCR assays including their modified versions have been developed for poxviruses of veterinary significance comprising the genera *Orthopoxvirus*, *Capripoxvirus* and *Parapoxvirus*. Considering certain disadvantages with conventional PCR methods, QPCR is the latest improvement in the PCR system. This technique is single-tube, closed system, which greatly reduces the chances of cross-contamination that results due to handling of PCR products in the conventional PCR methods. Moreover, the assay is about 100-times more sensitive and highly specific as compared to the latter and has an ability to detect as few as ten template molecules (Plumet and Gerlier, 2005). These methodologies use various approaches to alter the relative spatial arrangement of photon donor and acceptor molecule. The molecules can be anchored to the probes; primers or amplified products and these are normally selected so that amplification should increase the fluorescence.

QPCR method has been developed for orthopoxviruses (Sofi Ibrahim *et al.,* 2003; Nitsche *et al.,* 2004; Kulesh *et al.,* 2004; Olson *et al.,* 2004; Bhanuprakash *et al.,* 2006) and parapoxviruses (Gallina *et al.,* 2006; Nitsche *et al.,* 2006). Recently, Kallesh *et al.,* (2009), developed QPCR methods for potency estimation of live goatpox vaccine in single and combine vaccine formulations. This assay can also potentially be used for the potency estimation of live camel pox vaccine, as a quick tool for the quality control of vaccine batches.

Chapter III

Materials and Methods

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 camelpox 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 (BCS). For maintaining the cells, maintenance medium (MM) containing EMEM with 2% BCS was used.

3.1.3. Viruses

The seed camelpox virus (CMLV-I, P-49) collected from the scab material of camel infected with natural infection, maintained in the pox virus disease laboratory was used for the preparation of vaccine. The vaccine developed at this institute is currently undergoing field-testing in different parts of the country. Different batches of live attenuated camelpox vaccine were produced and used for the potency estimation by quantitative polymerase chain reaction (QPCR) assay. The sheeppox virus (SPPV-Srinagar, P-39), goatpox virus (GTPV- Uttarkashi, P-40) and buffalopox virus (BPXV-Vij/ 96, P-50) adapted in Vero cell line were used in the study for determining the specificity of the QPCR.

3.1.4. DNA isolation

DNA was extracted using Auprep Genomic DNA Isolation kit (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 of the PCR assay. T_4 DNA ligase and *Eco R*I (MBI Fermentas, Maryland, USA) were 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 Cloning, PCR and QPCR

Name of the Primer	Orientation	Sequence (5'-3')	Length	Gene target
HA F	Forward	CCATTGGAAAAAACACAGTAC	21	Haemagglutinin
HA R	Reverse	CCAAATATATTCCCATAGTC	20	gene
CoPV-3	Forward	AGGGATATCAAGGAATGCGA	20	A-type inclusion
CoPV-4	Reverse	TCCATATCAGCATTGCTTTC	20	potein (ATIP) gene
CMLV C18L F	Forward	GCGTTAACGCGACGTCGTG	19	Ankyrin repeat
CMLV C18L R	Reverse	GATCGGAGATATCATACTTTACTTTAG	27	protein gene
SYBR Green Quantitative PCR		CMLV C18L F and C18L R		

3.1.7. Kits used in this study

a) QuantiTect SYBR Greeen PCR kit: QIAGEN [QIAGEN GmbH, D-40724 Hilden, Germany]

b) Genomic DNA extraction kit- AuPrep™GEN^{bt}DNA Extraction kit [Life Technologies India (Pvt) Ltd, New Delhi, India]

c) Min Elute Gel Extraction kit: QIAGEN [QIAGEN GmbH, D-40724 Hilden, Germany]

d) Plasmid extraction kit: AuPrep SPIN Miniprep Kit [Life Technologies India (Pvt) Ltd, New Delhi, India]

3.1.8. Equipments

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. Incubators

Sanyo CO_2 incubator-MC0175 (Sanyo Electric Co. Ltd., Japan)

vi. Spectrophotometer

Biomate5 (Thermospectronic, England)

vii. Other Equipments

- Vortex mixer, cyclomixer (Remi Instruments 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).

- Sonicator- Soniprep 150, (SANYO, Japan).
- Horizontal and Vertical Laminar Air Flow Cabinets (Klenzaids, India)
- Ultracentrifuge (Sorvall, USA). Water bath (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 Schott Duran, AG Mainz, Germany.

3.2. Methods

3.2.1. Revival and bulk production of camelpox vaccine in Vero cells

A working seed (Passage 50) was prepared using the vaccine seed virus held at P49. Vero cells were subcultured and allowed to form confluent monolayer in EMEM (Appendix) containing 10% FBS on a roller apparatus set at 0.5 rpm. After 48 hrs, the confluent monolayer was washed with pre-warmed serum-free EMEM. Then the cells were infected with CMLV-I virus at 0.01 multiplicity of infection (m.o.i.). Then the roller bottles were incubated at 37°C room incubator. The infected roller bottles were monitored for the appearance of cytopathic effect (CPE). After 36-48 hrs post infection (hpi) when 80 to 90% CPE was observed, infected cultures were subjected to a cycle of freezing and thawing to harvest the virus. To maintain a uniform virus titer, virus harvest from all the roller bottles were pooled and kept at –80°C until used.

3.2.2. Identity testing of CMLV-I vaccine virus

The presence of CMLV-I vaccine strain in the cell culture was confirmed by the formation of characteristic cytopathic effect (CPE), PCR and cloning and sequencing of selected genes.

(a) Cytopathic effect (CPE)

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

(b) Nucleic acid based detection

The vaccine virus was confirmed by conventional PCR as well as SYBR Green based real-time PCR amplification of viral DNA using specific primers.

(c) Cloning and sequencing of selected gene

For further confirmation regarding the identity of the vaccine virus preparation, the C18L gene (partial) was cloned to pGEM-T^{Easy} vector and sequenced.

3.2.3. Preparation of stabilizers

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

(i) Stabilizer I (LS stabilizer)

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

- 100 gram of lactalbumin hydrolysate (LAH, M/s Difco, USA) was taken in a flask.
- 200ml of HBSS (Appendix) was added to it and stirrered on a magnetic stirrer for 10 minutes.
- Then the flask was kept in a water-bath at 37°C until LAH was dissolved completely.
- After cooling the solution to room temperature, 200 gm of sucrose (M/s SRL, Pvt Ltd, India) was added and stirred on a magnetic stirrer.
- Finally, the volume was made up to 500 ml with HBSS and pH was adjusted at 7.2 with 0.1N NaOH.

The solution was filtered using 0.45ìm Seitz filter and stored at 4°C until use.

(ii) Stabilizer I (BUGS stabilizer)

BUGS (Buffered Gelatin and Sorbitol) stabilizer was prepared according to Mariner *et al.* (1990a) with modifications.

- 7.0 gm gelatin hydrolysate (Difco) and 7.0 gm D-sorbitol (Sigma) was taken in a flask.
- Potassium phosphate buffer, pH-6.2 (Appendix) was added to it to make the final volume of 200 ml.
- The solution was kept on magnetic stirrer until solutes dissolved completely.

- The stabilizer was then filtered with a sterile Seitz filter (pore size-0.45 µm) used.

(iii) Stabilizer III (TAA stabilizer)

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

Solution I

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

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

Solution II

$CaCl_2$. $2H_2O$	0.05 g
$MgSO_4$. $7H_2O$	0.0038 g

The contents were dissolved in 50 ml of 1X PBS and sterilized using 0.45ìm 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, 4.37 ml of stabilizer II mixed with 10 ml of freeze-thawed CMLV virus suspension.

3.2.3. Sterility test of stabilizers and vaccine

Sterility testing of the stabilizers and vaccine was done as per the methods described in OIE Manual of standards (Anon, 1996; OIE, 2008). Soya casein digest media (Appendix) was used for the purpose. Eight test tubes containing 5 ml of Soya casein digest media was used for each stabilizer. Stabilizer/ vaccine in 0.5 ml volume was added to four tubes, out of which two tubes each were kept at 25°C and 37°C for detection of fungal and bacterial contamination, respectively. Two tubes (without any 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.

3.2.4. Lyophilization

Lyophilization was carried out using Edwards Modulyo 4K freeze-drier. Vaccine and stabilizer were mixed in adequate volumes. Taking sterile precaution, 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 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 allow raise of vacuum in the shelf.
- Usually, the vacuum reaches a safe limit of $10^{-0.06}$ torr in 1 hr, which was 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 use.

3.2.5. Measurement of residual moisture

Residual moisture (RM) in the freeze-dried product was measured by thermo gravimetric method described by Worral *et al.* (2001). The procedure is briefly described below.

- 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

$$\text{Residual moisture (\%)} = \frac{(A - B - D) - (C - D)}{(A - B - D)} \times 100$$

3.2.6. Thermo-stability testing of CMLV vaccine

The stability of CMLV vaccine virus in the freeze-dried form as well as after reconstitution was studied at various temperatures viz., 4°C, 25°C, 37°C, and 45°C (Sarkar *et al.,* 2003)

3.2.6.1. Virus titration

Virus titration was carried out in 96 well micro titer plates using Vero cells (Mariner *et al,* 1990a). The procedure is briefly described below.

- Vero cells were subcultured and 100µl of the cell suspension were added to each well (10^6 cells/ml).
- The plates were incubated overnight at 37°C in the presence of 5% CO_2.
- The vaccine virus was diluted 10-fold in EMEM with 2% BCS.
- 100µl of virus suspension was added to each well keeping three replicates/dilution.
- Four wells were kept as control per plate.
- The plates were incubated at 37°C in the presence of 5% CO2.
- Reading was taken after 48 hrs of titration.
- End points were calculated (Reed and Muench, 1938).

3.2.6.2. Stability of freeze-dried vaccine

Sufficient number of freeze-dried vials of vaccine with each stabilizer was exposed at 4°C in a refrigerator, 25°C and 37°C in incubators and 45°C in a dry oven. Samples were taken from the refrigerator on monthly intervals (1, 2, 3, 4 and 5th months), from 25°C on days 15, 30, 45, 60, 75, 90, 105, 120, 135 and 150; from 37°C and 45°C on days interval

upto 14 days. Exposed samples were reconstituted with 1 ml of serum free EMEM and titrated in Vero cells. For each exposure, two samples were titrated and their average log titer was calculated (Reed and Muench, 1938).

3.2.6.3. Stability of reconstituted vaccine

Freeze-dried vials of each stabilizer were reconstituted with four diluents (1X PBS, 0.85% NaCl, distilled water and 1M $MgSO_4$). For each stabilizer, three vials were taken and reconstituted separately with 100 ml each of four diluents as each vial contains 100 doses of vaccine. Reconstituted vaccines were titrated immediately (0 hour) to study immediate loss upon dilution and then exposed to 4°C (ice), 25°C, 37°C and 45°C after dividing in 15 ml translucent plastic centrifuge tubes. Samples from 37°C and 45°C temperature were taken out at 6 hourly intervals up to 60 hours and titrated immediately, where as samples from 25°C and 4°C were taken at days interval and titrated upto 10 days.

3.3. Density gradient purification of camelpox virus

Sucrose gradient purification of CMLV was carried out to concentrate and purify the camelpox virus.

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 centrifugations 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 –80^0 till use.

3.3.1. DNA extraction

DNA extraction was carried out using Genomic DNA extraction kit- AuPrep GEN DNA Extraction kit [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 as below:

i. 20µl Proteinase K and 200µl 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 hr 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 9,300xg for 60 sec at room temperature. Flow-through was discarded.

iv. The column was then placed in a clean 2ml collection tube and washed twice with 500µl of WS Buffer (supplied with the kit) and centrifuged at 6,000xg for 2 min and flow-through was discarded.

v. After discarding the flow-through, the column was dry spun for 3 min at full speed to completely dry the matrix.

vi. The dried column was then transferred to a new 1.5ml microcentrifuge tube and the DNA was eluted with 50µl of pre-warmed TE buffer by centrifuging at 10,000xg for 2 min and stored at –80^0 till use.

3.3.2. Polymerase Chain Reaction (PCR)

The PCR reaction was carried out in standard 50 µl reaction for C18L gene (encoding ankyrin repeat protein) following standard protocols. A typical PCR reaction contained the following reagents (Invitrogen, Carlsbad, California, USA) was performed in a 0.2ml PCR tube.

Components (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
Taq 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 spun briefly. The tubes were then placed in a thermocycler. PCR conditions for the amplification of the target gene using both sets of primers (C18L F/C18L R) are mentioned in Table 2.

Table 2.Thermal conditions used for amplification of target genes

Primers used	Thermal Profile						Expected size of amplicons
	1	2	3	4	5	6	
HA F HA R	95°C, 3 min	94°C, 1 min	51°C, 1 min	72°C, 1 min	34 cycles from steps 2 to 4	72°C, 10 min	1185 bp
CoPV-3 CoPV-4	95°C, 3 min	94°C, 1 min	52°C, 1 min	72°C, 1 min	34 cycles from steps 2 to 4	72°C, 10 min	564 bp
CMLV C18L F CMLV C18L R	95°C, 3 min	94°C, 30 sec	60°C, 30 sec	72°C, 30 sec	34 cycles from steps 2 to 4	72°C, 10 min	243 bp

3.3.3. Agarose gel electrophoresis of amplicons

1. 1.5% molten agarose (Sigma-Aldrich, St. Louis, USA) was prepared in TAE buffer (Appendix) in microwave oven and allowed to cool to about 50°C.

2. 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 electrophoresis apparatus and then comb was inserted. Gel was allowed to solidify at room temperature for 20 min.

3. Electrophoresis tank was filled with TAE buffer to submerge the gel 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.

3.3.4. Purification of PCR product

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 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 water-bath 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 12,000xg 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 12,000xg 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.

3.3.5. 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:

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

(i) Ligation of the PCR products into the plasmid vector

The PCR products were ligated with pGEM-T^{Easy} plasmid 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.

(ii) 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 A_{600} nm (usually for 3-4 h).

v. The culture was chilled on ice and the cells were harvested by centrifuging at 6000xg for 15 min at 4°C.

vi. The cell pellet was resuspended in 20 ml (1/10th volume) ice-cold solution of sterile 100mM $CaCl_2$ and was kept on ice for 30 min.

vii. The cells were again pelleted by centrifuging at 6000xg for 15 min at 4°C.

viii. Finally, the pellet was resuspended in 1.75 ml of ice-cold 10mM $CaCl_2$ 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.

(iii) 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 and the tube was flicked gently for homogenous mixing and was kept in ice for 20 min.

iii. The cells were then heat-shocked at 42°C for 45-50 sec in a water-bath and then immediately transferred on ice and kept for 2 min.

iv. 850µl of SOC (appendix) media 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.

(iv) 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.

3.3.6. Isolation of plasmid

i. 5.0 ml of overnight culture (from previous step) was taken in a 2.0 ml microcentrifuge tube and centrifuged at 8,000xg 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 13000xg 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 8000xg. The flow-through was discarded.

vi. The column was washed once with 0.5 ml WF Buffer by centrifuging for 60 sec. The flow-through was discarded.

vii. Then the column was washed once with 0.7 ml WS Buffer by centrifuging for 60 sec and the flow-through was discarded.

viii. The column was centrifuged at full speed for another 3 min to remove residual ethanol.

ix. And then the column was placed onto a fresh 1.5 ml microcentrifuge tube, and 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.

x. The eluted DNA was preserved at -20°C for future use.

3.3.7. 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.

Components	**Amount (volume)**
10X Buffer	1.5µl
EcoRI	1.0µl (10U)
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).

3.3.8. 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 isolates of camelpox viruses available in the GenBank database. Sequence identities were determined using the programm MegAlign of the Lasergene 6.0 software (DNASTAR Inc., Madison, USA).

3.3.9. SYBR green dye based QPCR

The SYBR green dye based QPCR reaction was carried out for C18L gene target using a reaction mixture of 50µl. A typical PCR reaction was made according to the protocol given by the Qiagen manufacturer's protocols with slight modification.

Components (stock)	Amount (volume)
Template DNA	2.0 µl
Forward primer (10 pmol)	1.0 µl
Reverse primer (10pmol)	1.0 µl
Premix (2X QuantiTect SYBR Greeen master mix)	25.0 µl
Nuclease free water	21.0 µl
Total reaction volume	50.0 µl

SYBR Green dye: Excitation maximum and emission maximum are 494 nm 521 nm wavelengths respectively. In QPCR the reading was taken at 521nm at end point.

Table. 3. PCR conditions optimized for SYBR green dye based QPCR

Primers used	Steps of thermal cycling							Expected size of amplicons
	1	2	3	4	5	6	7	
CMLV C18L F CMLV C18L R	95°C 10 min	94°C 30 sec	60°C* 30 sec	72°C* 1 min	95°C 1 min	55°C 30 sec	95°C 30 sec	243 bp
		40 cycles			Dissociation curve**			

* End point fluorescent reading in each cycle

** All point fluorescent reading in each cycle

3.4. Potency estimation of live attenuated camelpox vaccine by QPCR

3.4.1. Establishment of standard reference sample

CMLV-I (P-50) batch of live attenuated camelpox vaccine produced at poxvirus disease laboratory, Division of Virology was taken as for reference purpose. The reference vaccine was assigned with the $TCID_{50}$ by virus titration in confluent Vero cell monolayer seeded in 96 well plate and vaccine virus was stored in – 20°C until further use. The titer of virus was rechecked periodically to know storage effects on the vaccine virus titre. The procedure in brief is as follows.

i. The 10-fold dilution of reference vaccine virus was carried out in EMEM with 1% NBCS.

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. The appearance of CPE in all the wells was recorded on 36-48 hours post infection (hpi).

v. 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.

vi. Plate was rinsed with distilled water to remove the stain completely and air dried.

vii. 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 $TCID_{50}$ was calculated to assign the titer of virus for the reference vaccine batch.

3.4.2. Estimation of critical time for harvest

The critical time point of harvest for cells post infection to estimate the live viral particles in given test vaccine batch was established. This was carried out by infecting the different dilutions of camelpox virus to confluent Vero cell monolayer followed by harvesting at different hour intervals and carrying out QPCR upon extracting the DNA from each dilution. The detailed procedure is as follows.

i. CMLV-I, P-50 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 plates having a confluent Vero cell monolayer and the plates were incubated at 37°C for 1hour.

iii. Then the 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 CO_2 incubator maintained at 37°C.

iv. Each plate was harvested at 6 hours interval and DNA was isolated after repeated freeze.

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 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.

3.4.3. 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 were diluted serially ten-fold 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 36-hour p.i. 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 camelpox vaccine.

3.4.5. Statistical analysis

The degradation values of camelpox 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 $TCID_{50}$. For student's t-distribution, the standard table was referred from Bailey (1995).

Chapter IV

Results

4.1. Revival, bulk production and freeze-drying of camelpox vaccine virus

As mentioned in Materials and Methods, the camelpox vaccine virus (CMLV-1) at passage level P49 was revived in Vero cells. Bulk production of the vaccine virus was carried out in roller flasks having 24 hour old confluent Vero cell monolayer. Characteristic CPE was initiated within 24 hours and more than 80% CPE appeared after 48 hours post infection (hpi). Then the infected cells were harvested by repeated freezing and thawing for three times.

There were four lots of freeze-dried vaccine containing various stabilizers produced as per standard protocols. The titre of each batch before and after lyophilization and other criteria which attribute to the quality of vaccine are given in Table 1. As shown in the table, the initial titre of all the batches of vaccine was around 7.00 $\log_{10}$ $TCID_{50}$ per ml. The loss of virus titre during similar freeze-drying conditions was maximum with BUGS stabilizer. The vaccine which contains the BUGS stabilizer was found to have the least residual moisture (RM) and the vaccine stabilized with TAA stabilizer had a maximum RM than with LS stabilizer (Table 1).

4.2. Identity of the vaccine virus

The vaccine virus (CMLV-1) used in the present study was propagated in Vero cells and tested for its identity based on characteristics CPE, PCR (both conventional and real time) as well as by cloning and sequencing of selected target genes viz., *A-type inclusion protein (ATI) gene, haemagglutinin (HA) gene and ankyrin repeat protein gene (C18L)*. Virus induced CPE was characterized by rounding, vacuolization, cytolytic changes and sometimes multinucleated giant cell formation, and syncytia. CPE was initiated by 24 hpi and

Table 1. Comparative quality of camelpox vaccine lyophilized with various stabilizers.

Vaccine Batches	Stabilizer	Titer ($log_{10}TCID_{50}$/ml)		Loss during lyophilization ($log_{10}TCID_{50}$/ml)	Residual moisture (%)	Appearence
		Before lyophilization	After lyophilization*			
01/091209	**LS**	6.75	6.53	0.22	3.28	Tablet form
	BUGS	6.75	6.23	0.52	1.78	White tablet form
	TAA	6.75	6.53	0.22	3.40	Pale pink granular form
02/161209	**LS**	7.25	7.00	0.25	3.48	Tablet form
	BUGS	7.25	6.67	0.58	0.64	White tablet form
	TAA	7.25	6.77	0.48	4.54	Pale pink granular form
03/311209	**BUGS**	6.75	6.50	0.25	1.20	White tablet form
	TAA	6.75	6.67	0.08	3.38	Pale pink granular form
04/060110	**LS**	7.25	6.75	0.50	3.29	Tablet form
	BUGS	7.25	6.50	0.75	1.63	White tablet form
	TAA	7.25	6.75	0.50	3.72	Pale pink granular form

LS : Lactalbumin hydrolysate and sucrose
BUGS : Buffered gelatin and sorbitol
TAA : Trehalose dehydrate, amino acids and divalent cations
* The freeze-dried vaccines were reconstituted with EMEM; a dilution factor of 0.3 log_{10} $TCID_{50}$ was added to the titer in each vial as each vial had contained 0.5 ml of original undiluted vaccine virus.

progressed to 80% by 48 hpi (Fig. 1A and 1B). In conventional PCR, genus specific *ATI* as well as *HA genes* were amplified by using respective and specific primer sets which gave 564 and 1185 bp amplicons, respectively. As so, in the species specific C18L gene based conventional PCR, CMLV vaccine virus gave a product size of 243 bp (Fig. 2). Further, they were confirmed as CMLV by amplification of *C18L gene* in SYBR green based real time PCR. The amplification plot with respect to vaccine virus is shown along with related virus and non-template controls which gave no amplification signal (Fig. 3). As well, amplicons of C18L gene of the vaccine virus was cloned into pGEM-T^{Easy} vector and sequenced commercially. The nucleotide sequences of the C18L gene of the vaccine virus (Table 2) was compared with that of the sequences available in the GenBank database. Further, phylogenetic analysis of this gene sequences showed that the C18L gene sequences of the vaccine virus used in the present study are clustered with other previously published camelpox virus sequences (Fig. 4). The aforementioned tests were found enough to confirm the identity of the vaccine virus as CMLV and then the vaccine virus is evaluated for its stability and potency.

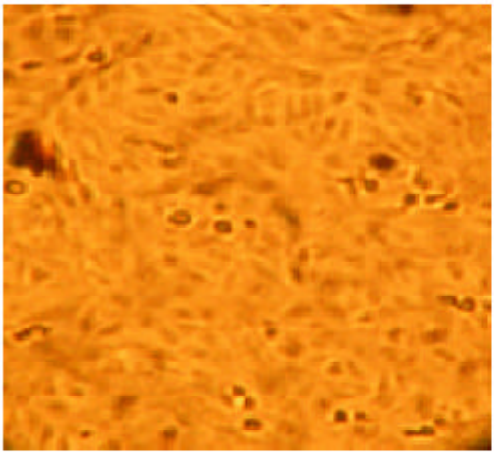
4.3. Thermo-stability evaluation of live attenuated camelpox vaccine

4.3.1. Thermo-stability of freeze-dried vaccine

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

4.3.1.1. Stability at 4°C

Freeze-dried vaccine vials were taken out from the refrigerator on monthly intervals up to 5 months and then titrated. Titres obtained for vaccines containing various stabilizers are tabulated in Table 3. Regression analysis was done based on the values and degradation curves are depicted in Fig. 5.There was no significant degradation in vaccines with all stabilizer combinations. The vaccines with all stabilizers although showed a declining trend over the period of exposure, the decline was not significant based on student's t-test. From the data, it is clear that at 4°C, vaccines containing LS or TAA have superior keeping quality than BUGS stabilizer.



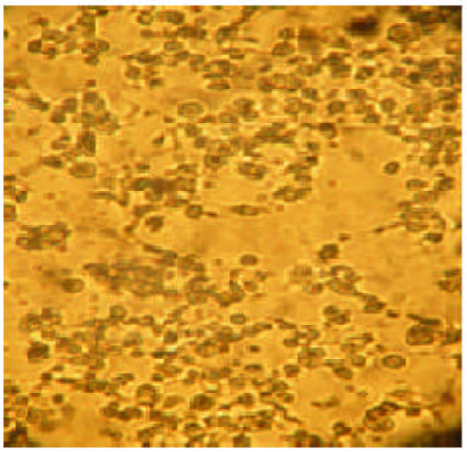
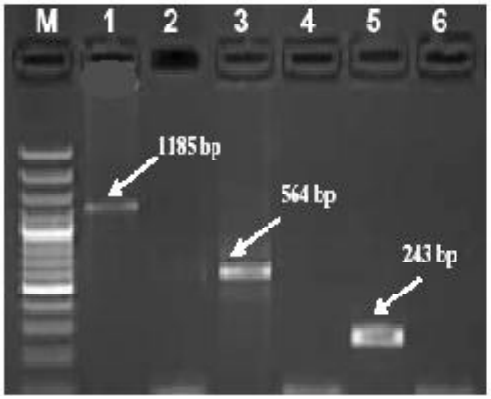


Fig. 1A. **Fig. 1B.**

Fig. 1A. Healthy Vero cells

Fig. 1B. Vero cells infected with camelpox virus (CMLV-1) showing charactreristics cytopathic effects viz., cell rounding, vacuolization, cytolytic changes and sometimes multinucleated giant cell formation.



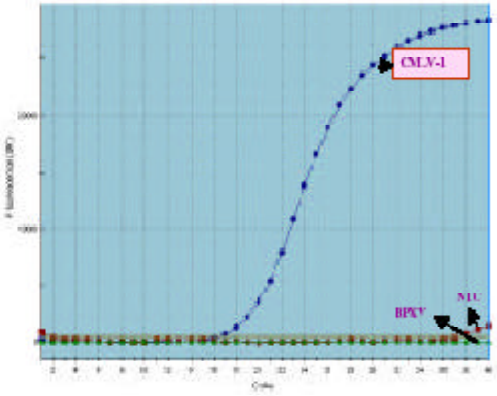


Fig. 2. **Fig. 3.**

Fig.2. Conventional gel based PCR: Agarose gel electrophoresis of polymerase chain reaction products of CMLV-1 vaccine virus. lane M: DNA size marker (100-bp ladder plus); lane 1: HA PCR amplicon; lane 3: ATI PCR amplicon; lane 5: C18L PCR amplicon and lanes 2, 4 and 6: Non template controls

Fig.3. SYBR Green diagnostic quantitative polymerase chain reaction for specific detection and quantification of camelpox virus (CMLV) DNA showing specific amplification from CMLV-1 and no such signal from BPXV isolate and NTC

Table 2. NCBI BLAST result of CMLV-1 virus ankyrin repeat protein (C18L) gene amplicon sequence

Accession Number	Description	Query coverage (%)	Maximum identity (%)
EF205282	Camelpox virus isolate CMLV-1 C18L-like protein gene, partial cds	100	100
EF592574	Camelpox virus isolate CMLV-Hyd C18L-like protein gene, partial cds	100	100
GQ465934	Camelpox virus isolate DEL09 C18L-like protein (C18L) gene, partial cds	100	100
GQ465933	Camelpox virus isolate JSL09 C18L-like protein (C18L) gene, partial cds	100	100
GQ465932	Camelpox virus isolate BAR09 C18L-like protein (C18L) gene, partial cds	100	100
EF205283	Camelpox virus isolate CMLV-2 C18L-like protein gene, partial cds	100	99
AF438165	Camelpox virus M-96 from Kazakhstan, complete genome	98	100
AY009089	Camelpox virus CMS, complete genome	98	100

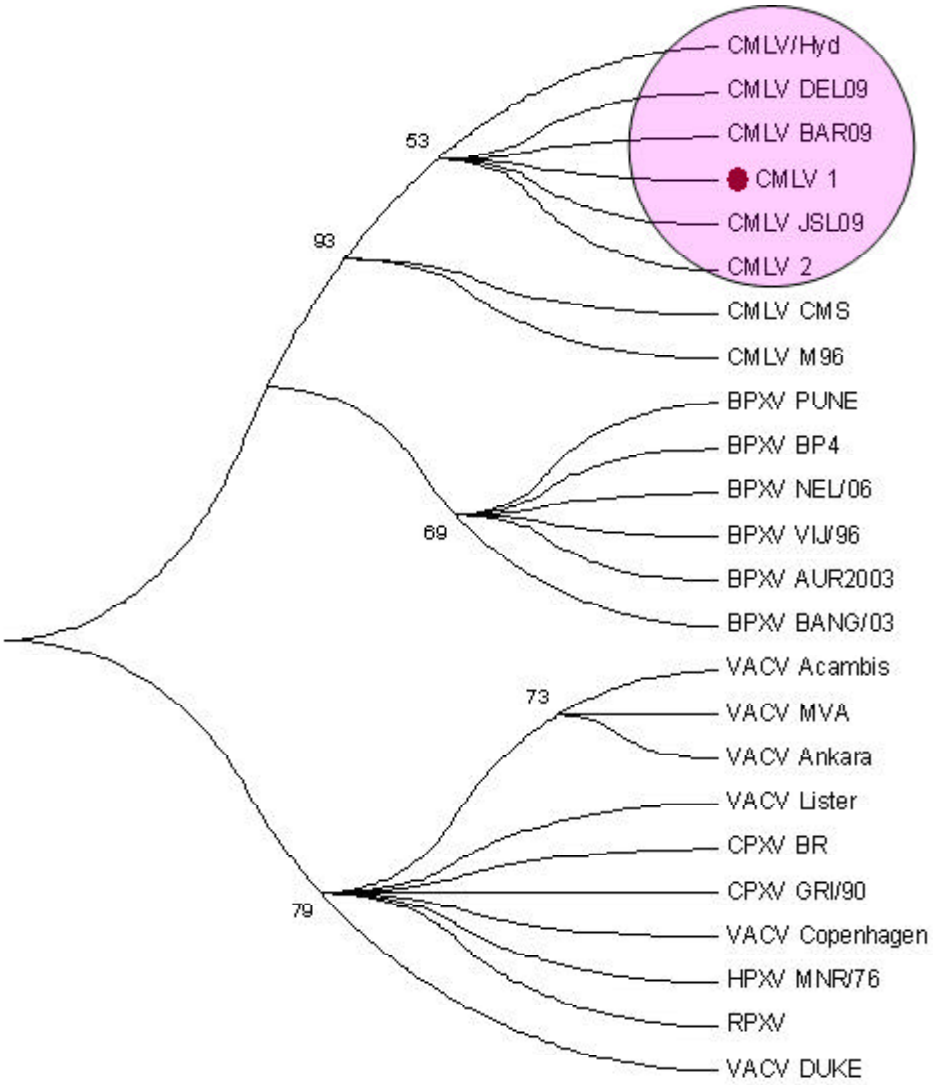

CMLV/Hyd
CMLV DEL09
CMLV BAR09
CMLV 1
CMLV JSL09
CMLV 2
CMLV CMS
CMLV M96
BPXV PUNE
BPXV BP4
BPXV NEL/06
BPXV VIJ/96
BPXV AUR2003
BPXV BANG/03
VACV Acambis
VACV MVA
VACV Ankara
VACV Lister
CPXV BR
CPXV GRI/90
VACV Copenhagen
HPXV MNR/76
RPXV
VACV DUKE
53
93
69
73
79

Table 3. Comparison of degradation values of camelpox vaccine containing various stabilizers at 4°C

		Titre ($log_{10}TCID_{50}$)		
		LS	BUGS	TAA
Months post exposure	0	6.53	6.53	6.53
	1	6.50	6.33	6.50
	2	6.50	6.33	6.33
	3	6.33	6.00	6.50
	4	6.33	6.00	6.33
	5	6.33	6.00	6.33
Shelf life[1] (Months)		34.88	13.44	44.11
Half life[2] (Months)		6.86	2.75	8.82

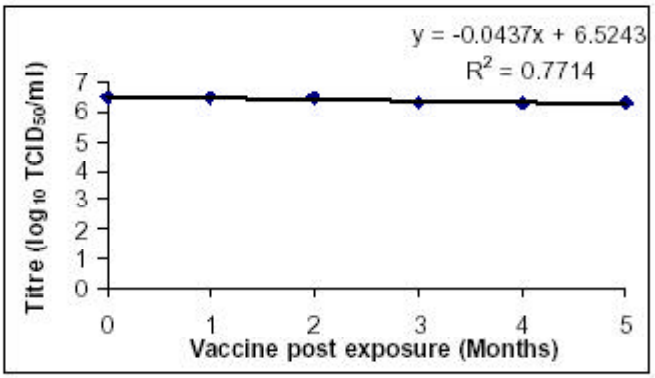
Table 4. Comparison of degradation values of camelpox vaccine containing various stabilizers at 25°C

		Titre ($log_{10}TCID_{50}$)		
		LS	BUGS	TAA
Days post exposure	0	6.53	6.5	6.53
	15	6.33	6.33	6.50
	30	6.33	6.00	6.33
	45	6.33	6.00	6.50
	60	6.00	6.00	6.33
	75	5.67	5.50	5.67
	90	5.67	5.50	6.00
	105	5.67	5.33	5.67
	120	5.67	5.50	5.67
	135	5.67	5.50	5.67
	150	5.50	5.33	5.50
Shelf life[1] (Days)		214.23	168.53	214.79
Half life[2] (Days)		44.11	43.47	40.54

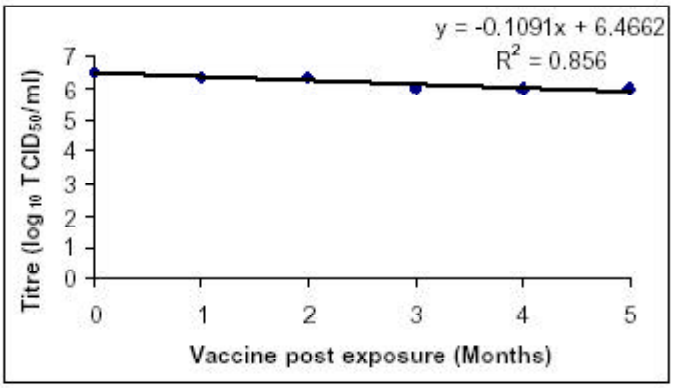
LS : Lactalbumen hydrolysate andsucrose
BUGS : Buffered gelatin and sorbitol
TAA : Trehalose dehydrate, amino acids and divalent cations

[1] Time required to reach 5 $log_{10}TCID_{50}$ in a 100 dose vaccine preparation, calculated from regression equation.
[2] Time required for loss of half the original titre i.e., 0.30 $log_{10}TCID_{50}$ based on degradation constant.

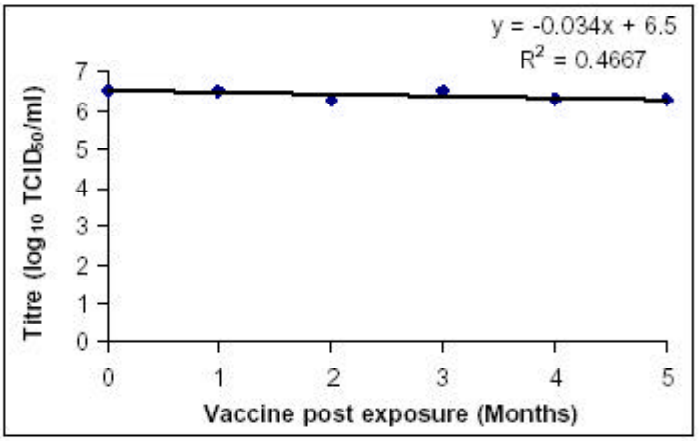
Fig. 5. Degradation curves for lyophilized camelpox vaccine with various stabilizers at 4 °C



a) Lactalbumin hydrolysate and sucrose



b) Buffered gelatin and sorbitol



c) Trehalose dehydrate, amino acids and divalent cations

4.3.1.2. 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 5 months and sampling at 15 days interval. The infectivity titres, thus obtained for each sample of the vaccine virus are given in Table 4. Regression analysis was done based on these values and the degradation curves are depicted in Fig. 6. All three stabilizers could maintain infectivity titre for 150 days. From the regression equations, shelf-life and half-life were calculated. Both LS and TAA stabilizers had shown better infectivity titre compared to the remaining one, indicating their superiority over BUGS stabilizers. Although the shelf-life of TAA stabilized vaccine was comparable with LS stabilized vaccine, half-life incase of LS stabilizer indicates its marginal superiority at a storage temperature of 25°C.

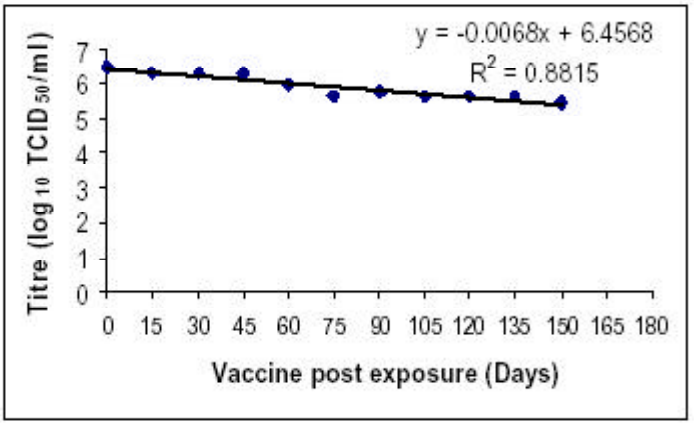
4.3.1.3. Stability at 37°C

Samples taken from 37°C at different time intervals studied for infectivity titre of the vaccine virus are tabulated in Table 5. The titres were subjected to regression analysis and the degradation curves are depicted in Fig. 7. Based on 15 days study period, it was found that infectivity titres at 37°C for the vaccine virus containing LS and TAA stabilizers were comparable and BUGS showed less infectivity titres. However, vaccines with all three stabilizers were able to maintain the infectivity titre of 10^3 $\log_{10}TCID_{50}$ per dose for the study period. In contrast to 25°C, TAA stabilizer showed longer half-life and shelf-life than LS stabilizer at 37°C, and the BUGS stabilizer showed the least.

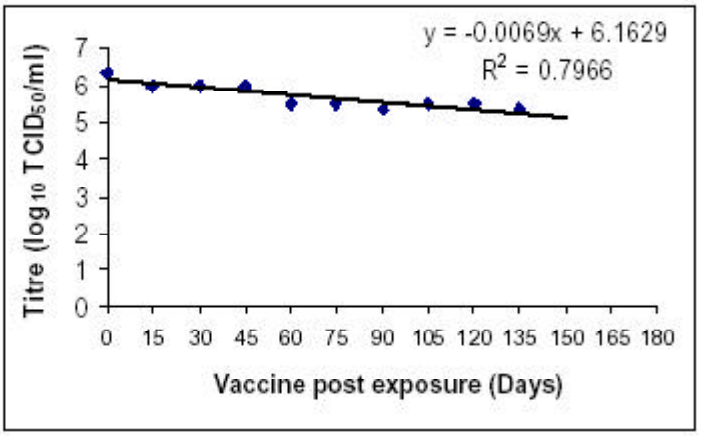
4.3.1.4. Stability at 45°C

Freeze-dried vials exposed at 45°C were sampled at days interval and then titrated. Infectivity titres obtained are given in Table 6. The titre values were subjected to regression analysis and the degradation curves are depicted in Fig. 8. At 45°C storage temperature which is commonly observed during summer in several parts of India, there was decrease in infectivity titres of the vaccine with all the three stabilizers. However, BUGS stabilizer showed more loss in infectivity with least half-life and it could not maintain the recommended titre for more than 10 days. Although, the other two stabilizers were shown to have the required infectivity titre for the whole study period of 15 days, TAA stabilized vaccine showed longer half- life and shelf- life than the LS stabilizer.

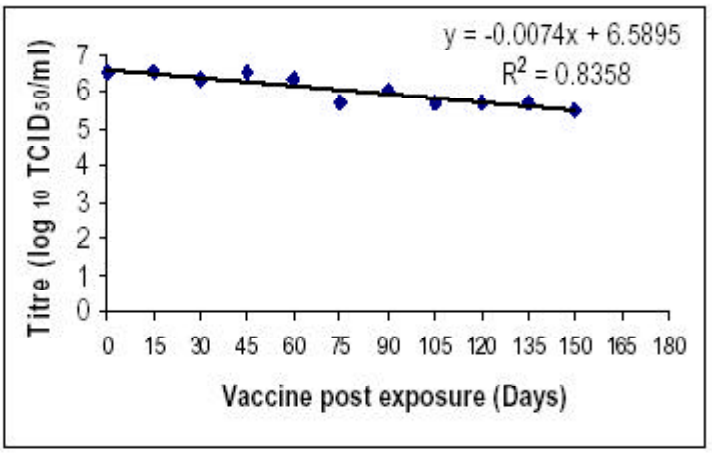
Fig. 6. Degradation curves for lyophilized camelpox vaccine with vario stabilizers at 25°C



a) Lactalbumin hydrolysate and sucrose



b) Buffered gelatin and sorbitol



c) Trehalose dehydrate, amino acids and divalent cations

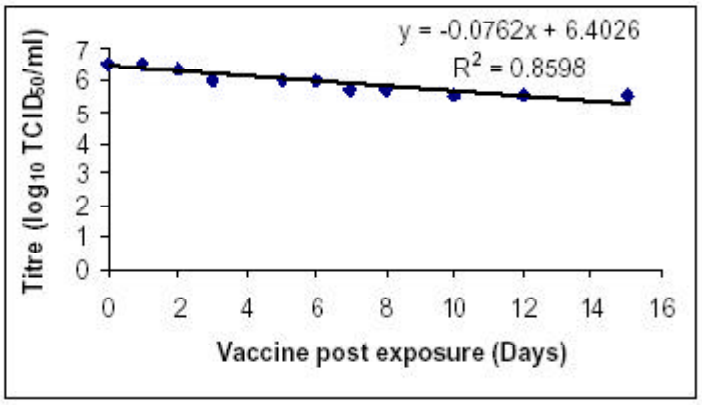
Table 5. Comparison of degradation values of camelpox vaccine containing various stabilizers at 37°C

		Titre ($log_{10}TCID_{50}$)		
		LS	**BUGS**	**TAA**
Days post exposure	0	6.53	6.50	6.53
	1	6.5	6.33	6.00
	2	6.33	5.67	6.00
	3	6.00	5.67	6.00
	5	6.00	5.67	6.00
	6	6.00	5.67	6.00
	7	5.67	5.50	5.67
	8	5.67	5.50	6.00
	10	5.50	5.33	5.50
	12	5.50	5.33	5.50
	15	5.50	5.00	5.50
Shelf life[1] (Days)		18.40	14.32	22.08
Half life[2] (Days)		3.93	3.70	5.39

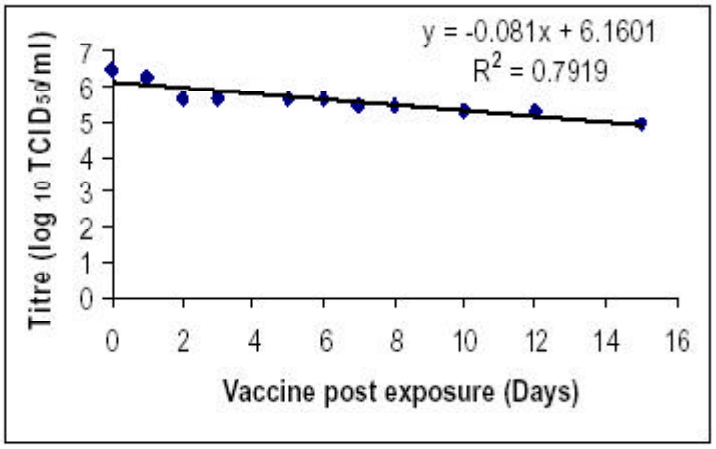
LS : Lactalbumen hydrolysate andsucrose
BUGS : Buffered gelatin and sorbitol
TAA : Trehalose dehydrate, amino acids and divalent cations

[1] Time required to reach 5 $log_{10}TCID_{50}$ in a 100 dose vaccine preparation, calculated from regression equation.
[2] Time required for loss of half the original titre i.e., 0.30 $log_{10}TCID_{50}$ based on degradation constant.

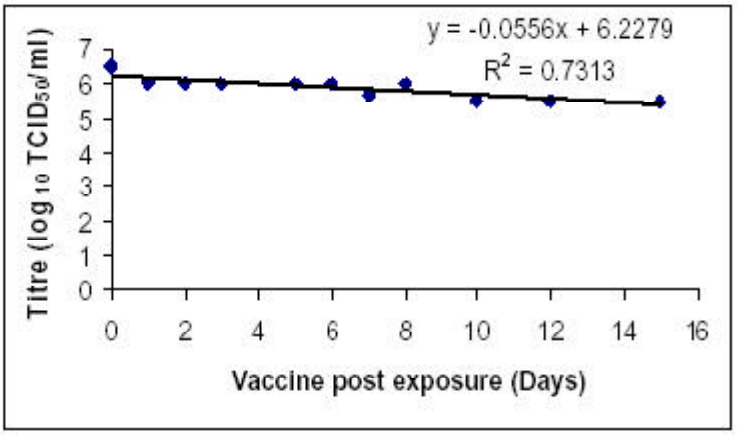
Fig. 7. Degradation curves for lyophilized camelpox vaccine with variou stabilizers at 37°C



a) Lactalbumin hydrolysate and sucrose



b) Buffered gelatin and sorbitol



c) Trehalose dehydrate, amino acids and divalent cations

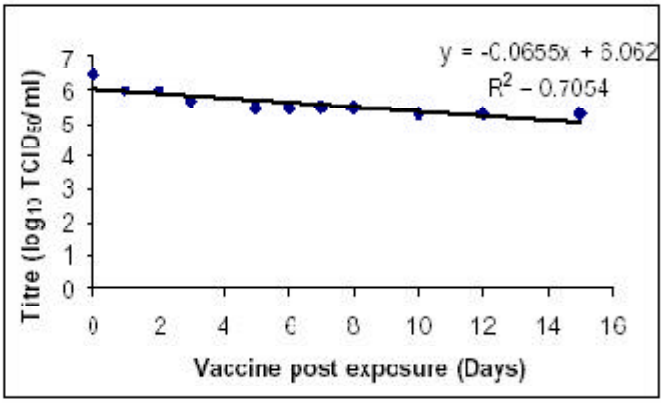
Table 6. Comparison of degradation values of camelpox vaccine containing various stabilizers at 45°C

		Titre ($log_{10}TCID_{50}$)		
		LS	BUGS	TAA
Days post exposure	0	6.53	6.50	6.53
	1	6.00	6.00	6.00
	2	6.00	5.67	6.00
	3	5.67	6.00	6.00
	5	5.50	5.50	5.67
	6	5.50	5.50	5.67
	7	5.50	5.33	5.67
	8	5.50	5.33	5.67
	10	5.33	5.00	5.50
	12	5.33	4.67	5.50
	15	5.33	4.67	5.50
Shelf life[1] (Days)		16.21	10.65	20.38
Half life[2] (Days)		4.58	2.74	5.36

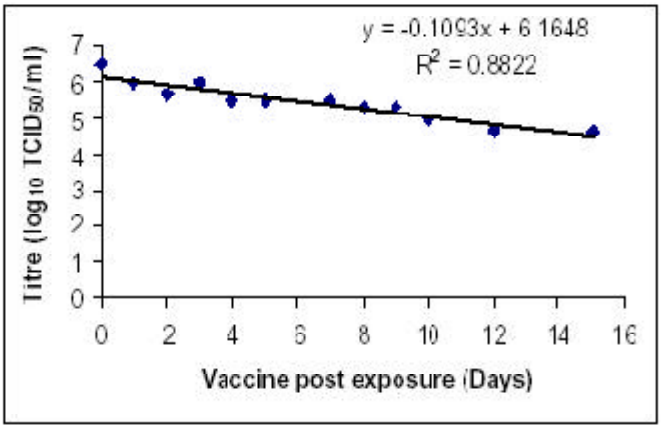
LS : Lactalbumin hydrolysate and sucrose
BUGS : Buffered gelatin and sorbitol
TAA : Trehalose dehydrate, amino acids and divalent cations

[1] Time required to reach 5 $log_{10}TCID_{50}$ in a 100 dose vaccine preparation, calculated from regression equation.
[2] Time required for loss of half the original titre i.e., 0.30 $log_{10}TCID_{50}$ based on degradation constant.

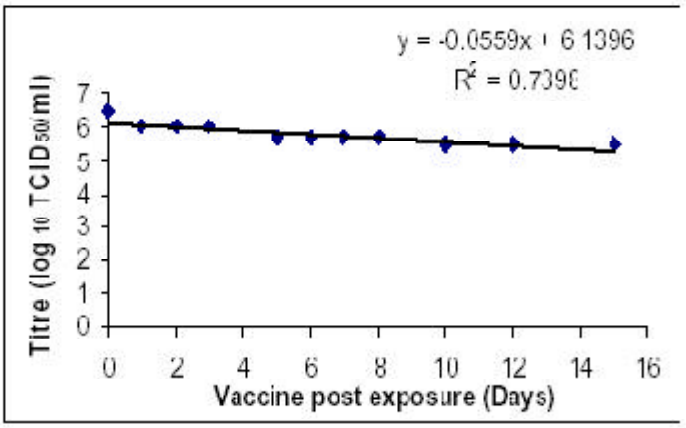
Fig. 8. Degradation curves for lyophilized camelpox vaccine with various stabilizers at 45°C



a) Lactalbumin hydrolysate and sucrose



b) Buffered gelatin and sorbitol



c) Trehalose dehydrate, amino acids and divalent cations

4.3.2. Thermo-stability of reconstituted camelpox vaccine

Lyophilized vaccines stabilized with different stabilizers were reconstituted using 100 ml of each of the four different diluents to provide 100 doses per vial and exposed at 4°C, 25°C for 60 hrs and at 37°C and 45°C for 10 days. Infectivity titres of samples diluted in various diluents exposed at each temperature are shown in Tables 7-10. The regression analysis of the reconstituted vaccines at various temperatures is represented graphically in Fig. 9-20.

There was immediate loss in the vaccine titre (0.25 $\log_{10}TCID_{50}$) upon dilution in case of BUGS stabilizer diluted with distilled water. It was revealed that at 4°C, in the vaccines with all three stabilizer combinations diluted with four different diluents, there was no significant loss of infectivity titre over the sampling period of 10 days. At 25°C, the vaccines could maintain the required infectivity titre (10^3 $\log_{10}TCID_{50}$) with all the diluents for 5 days and thereafter, there was a reduction in infectivity titre with all diluents excluding PBS. At higher temperatures (both 37°C and 45°C), the required titre was maintained for 60 hours study period by all the diluents except 1M $MgSO_4$. At all the temperatures studied, vaccines diluted with PBS showed better stability over other three diluents with all stabilizer combinations. Almost, LS stabilized vaccines showed comparable results to vaccines with TAA stabilizer. 1M $MgSO_4$ was found to be a less suitable diluent in all the cases, particularly with BUGS stabilized vaccines in which it could not maintain the titre even for 6 hours at higher temperatures. Overall, vaccines stabilized with both LS and TAA stabilizers diluted with PBS diluent showed better stability and the BUGS stabilizer was found to be a poor stabilizer for camelpox vaccine in the present study.

4.4. Potency estimation of live attenuated camelpox vaccine by real-time PCR

4.4.1. SYBR Green based Q-PCR

The optimized SYBR Green based real-time PCR assay was employed to estimate the potency of live attenuated camelpox vaccine. This was carried out with CMLV C18L-F and CMLV C18L-R primer set, to get a typical dissociation curve with the melting temperature (Tm) of 77.6°C which is specific for CMLV. The specificity was tested against Orf virus (*Parapoxvirus)*, BPXV *(Orthopoxvirus)* and SPPV and GTPV (*Capripoxviruses)* and found to be specific for CMLV only (Fig. 21). Further, the sensitivity of the SYBR green

Table 7. Thermo-stability of camelpox vaccine stabilized with various stabilizers at 4 °C after reconstitution with different diluents

		LS				BUGS				TAA			
		PBS	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**
UndilutedTiter		6.53	6.53	6.53	6.53	6.50	6.50	6.50	6.50	6.53	6.53	6.53	6.53
Loss on dilution		NIL	NIL	NIL	NIL	NIL	NIL	0.25	NIL	NIL	NIL	NIL	NIL
Days post exposure	1	4.50	4.25	4.50	4.50	4.50	4.50	4.25	4.50	4.50	4.50	4.25	4.50
	2	4.50	4.25	4.50	4.50	4.50	4.50	4.25	4.50	4.50	4.50	4.25	4.25
	3	4.50	4.50	4.50	4.25	4.25	4.50	4.25	4.25	4.50	4.50	4.25	4.25
	4	4.25	4.25	4.25	4.25	4.25	3.50	3.75	2.75	4.25	4.25	3.75	3.75
	5	4.50	4.25	4.25	4.25	4.25	4.25	4.25	4.25	4.25	4.25	4.25	3.75
	6	4.50	4.50	4.25	4.25	4.25	4.25	4.25	4.25	4.50	4.25	4.25	3.75
	7	4.50	4.50	4.50	4.25	4.50	4.25	4.25	4.25	4.25	4.25	4.25	3.75
	8	4.25	4.25	4.25	4.25	4.25	4.25	3.75	3.75	4.25	4.25	3.75	3.75
	9	4.25	3.75	3.75	3.75	4.25	3.75	3.75	3.75	3.75	3.75	3.75	3.75
	10	3.75	3.75	3.25	3.75	3.75	3.50	3.25	3.25	3.75	3.50	3.25	3.75
Degradation Constant (k)		NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

PBS- Phosphate buffer saline; **NaCl** - Normal saline solution; **DW**- Distilled water; **$MgSO_4$**- 1M $MgSO_4$

* Loss on dilution was calculated as the difference between the titer of the vaccine rehydrated in 1.0 ml of distilled water at 4°C and titer/ml of the vaccine rehydrated with 100 ml (100 doses) of corresponding diluents plus the dilution factor (1.7 $\log_{10}TCID_{50}$)

Table 8. Thermo-stability of camelpox vaccine stabilized with various stabilizers at 25 °C after reconstitution with different diluents

		LS				BUGS				TAA			
		PBS	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**
Undiluted Titer		6.53	6.53	6.53	6.53	6.50	6.50	6.50	6.5	6.53	6.53	6.53	6.53
Loss on dilution		NIL	NIL	NIL	NIL	NIL	NIL	0.25	NIL	NIL	NIL	NIL	NIL
Days post exposure	1	4.50	4.50	4.25	4.25	4.50	4.50	4.25	-	4.5	4.50	4.50	4.25
	2	4.50	4.50	3.75	3.75	4.50	4.50	4.25	-	4.25	4.25	4.50	3.75
	3	4.50	4.25	3.75	3.50	4.25	4.50	4.25	-	4.25	3.75	4.25	3.50
	4	4.25	4.25	3.75	3.50	3.75	3.50	3.75	-	3.75	3.75	4.25	3.25
	5	4.25	4.25	3.75	3.50	3.50	3.50	3.75	-	3.75	3.75	3.25	3.25
	6	4.25	3.75	3.50	3.25	3.50	3.50	3.75	-	3.50	3.50	2.50	2.50
	7	3.75	3.75	3.50	3.25	3.25	3.50	3.75	-	3.50	3.50	2.50	2.50
	8	3.75	3.75	3.25	2.75	3.25	3.25	3.50	-	3.50	3.25	2.50	2.50
	9	3.50	3.25	2.75	2.50	3.25	2.75	3.25	-	3.50	2.50	2.50	2.50
	10	3.25	2.75	2.50	2.50	2.75	2.50	2.75	-	3.25	2.50	2.50	2.75
Degradation Constant (k)		NSD	NSD	NSD	NSD	NSD	NSD	NSD	-	NSD	NSD	NSD	NSD

PBS- Phosphate buffer saline; **NaCl** - Normal saline solution; **DW**- Distilled water; **$MgSO_4$**- 1M $MgSo_4$
NSD- No significant degradation

* Loss on dilution was calculated as the difference between the titer of the vaccine rehydrated in 1.0 ml of distilled water at 4°C and titer/ml of the vaccine rehydrated with 100 ml (100 doses) of corresponding diluents plus the dilution factor (1.7 $\log_{10}TCID_{50}$)

Table 9. Thermo-stability of camelpox vaccine stabilized with various stabilizers at 37 °C after reconstitution with different diluents

		LS				BUGS				TAA			
		PBS	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**	**PBS**	**NaCl**	**DW**	**$MgSO_4$**
Undiluted Titer		6.53	6.53	6.53	6.53	6.50	6.50	6.50	6.5	6.53	6.53	6.53	6.53
Loss on dilution		NIL	NIL	NIL	NIL	NIL	NIL	0.25	NIL	NIL	NIL	NIL	NIL
Hours post exposure	6	4.50	4.50	4.25	4.25	4.50	4.50	4.25	-	4.50	4.50	4.25	4.25
	12	4.25	4.25	4.25	4.25	4.25	4.25	4.25	-	4.50	4.50	4.25	4.25
	18	4.25	3.75	4.25	4.25	4.25	3.75	4.25	-	4.50	4.25	4.25	4.25
	24	4.25	3.75	4.25	3.75	4.25	3.75	3.75	-	4.50	4.25	4.25	3.75
	30	4.50	3.75	3.75	3.75	4.50	3.75	4.25	-	4.25	4.25	3.75	3.25
	36	4.25	3.75	4.25	3.50	4.25	3.75	4.25	-	4.25	4.25	3.75	3.25
	42	4.25	3.75	3.75	3.25	4.25	4.25	3.75	-	4.50	3.75	3.50	2.75
	48	4.25	3.50	3.75	3.25	4.25	3.75	3.75	-	4.25	3.75	3.75	2.75
	54	3.75	3.50	3.50	3.25	3.75	3.50	3.75	-	4.25	3.75	3.50	2.75
	60	3.75	3.25	3.50	3.25	3.75	3.25	3.50	-	3.75	3.50	3.50	2.75
Degradation Constant (k)		NSD	NSD	NSD	NSD	NSD	NSD	NSD	-	NSD	NSD	NSD	NSD

PBS- Phosphate buffer saline; **NaCl** - Normal saline solution; **DW**- Distilled water; **$MgSO_4$**- 1M $MgSo_4$
NSD- No significant degradation

* Loss on dilution was calculated as the difference between the titer of the vaccine rehydrated in 1.0 ml of distilled water at 4°C and titer/ml of the vaccine rehydrated with 100 ml (100 doses) of corresponding diluents plus the dilution factor (1.7 $\log_{10}TCID_{50}$)

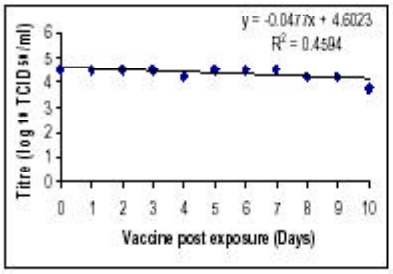
Table 10. Thermo-stability of camelpox vaccine stabilized with various stabilizers at 45 °C after reconstitution with different diluents

		Titer ($log_{10}TCID_{50}$)											
		LS				BUGS				TAA			
		PBS	NaCl	DW	$MgSO_4$	PBS	NaCl	DW	$MgSO_4$	PBS	NaCl	DW	$MgSO_4$
Undiluted Titer		6.53	6.53	6.53	6.53	6.5	6.5	6.5	6.5	6.53	6.53	6.53	6.53
Loss on dilution*		NIL	NIL	NIL	NIL	NIL	NIL	0.25	NIL	NIL	NIL	NIL	NIL
Hours post exposure	6	4.50	4.25	4.25	4.50	4.50	4.25	4.25	-	4.50	4.25	3.75	4.00
	12	4.50	4.25	4.25	3.75	4.50	4.25	4.25	-	4.50	4.25	3.75	3.75
	18	4.50	3.75	4.25	3.50	4.25	4.25	4.25	-	4.25	4.25	3.75	3.50
	24	4.50	4.25	4.25	3.25	4.50	3.75	4.25	-	4.50	3.75	3.50	3.25
	30	4.50	3.75	3.75	3.25	4.50	3.75	4.25	-	4.25	3.75	3.50	3.25
	36	4.50	3.75	3.75	3.25	4.25	3.75	3.75	-	4.25	3.75	3.25	2.75
	42	4.25	3.75	3.25	2.75	4.25	3.75	3.75	-	4.25	3.75	2.75	2.50
	48	4.25	3.50	3.50	2.75	4.25	3.50	3.50	-	3.75	3.25	2.75	2.50
	54	4.25	3.50	3.25	2.75	4.25	3.50	3.50	-	3.50	3.25	2.50	2.25
	60	3.75	3.25	3.25	2.75	4.25	3.25	3.25	-	3.50	3.25	2.50	2.25
Degradation Constant (k)		NSD	NSD	NSD	NSD	NSD	NSD	NSD	-	NSD	NSD	NSD	NSD

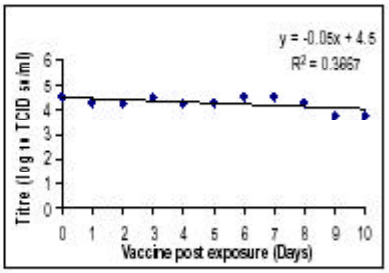
PBS- Phosphate buffer saline; **NaCl** - Normal saline solution; **DW**- Distilled water; **$MgSO_4$**- 1M $MgSo_4$
NSD- No significant degradation

* Loss on dilution was calculated as the difference between the titer of the vaccine rehydrated in 1.0 ml of distilled water at 4°C and titer/ml of the vaccine rehydrated with 100 ml (100 doses) of corresponding diluents plus the dilution factor (1.7 $log_{10}TCID_{50}$).

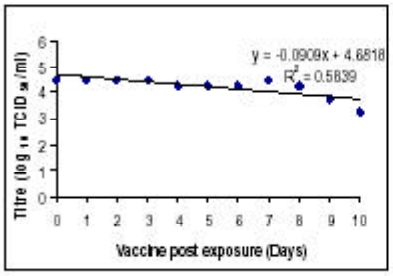
Fig 9. Degradation curves (4°C) for LS stabilized camelpox vaccine reconstituted v various diluents



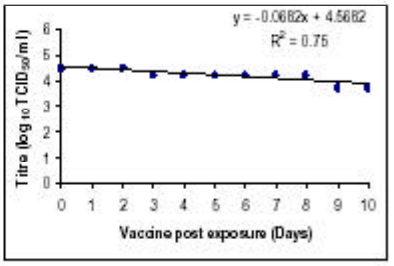
PBS



NaCl

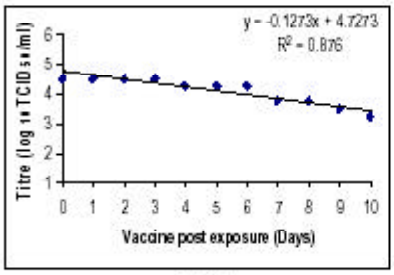


DW

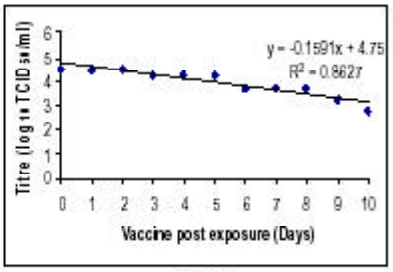


$MgSO_4$

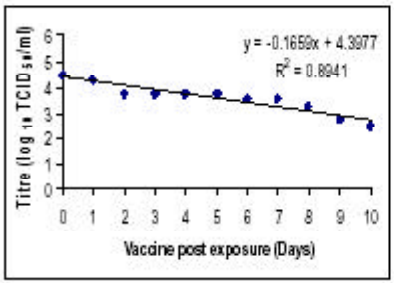
Fig 10. Degradation curves (25°C) for LS stabilized camelpox vaccine reconstitute with various diluents



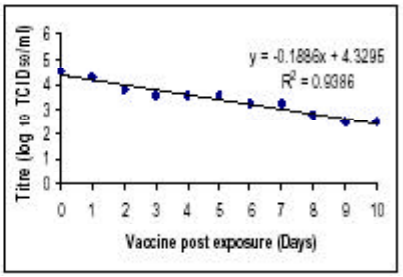
PBS



NaCl

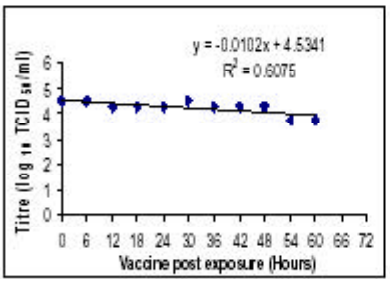


DW

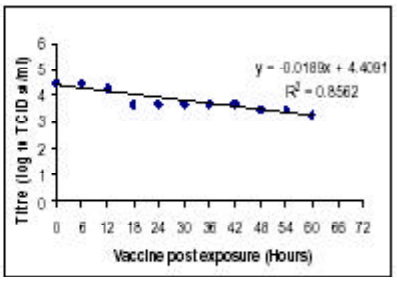


$MgSO_4$

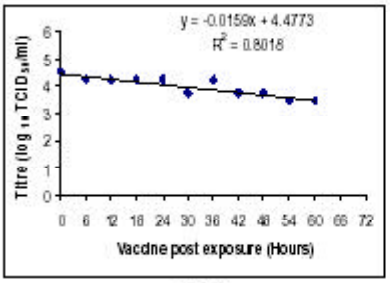
Fig 11. Degradation curves (37°C) for LS stabilized camelpox vaccine reconstitute with various diluents



PBS



NaCl



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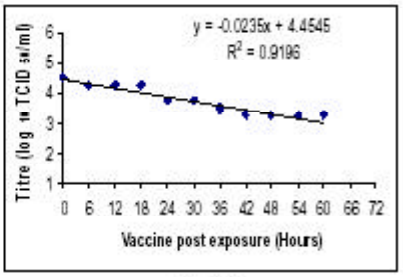
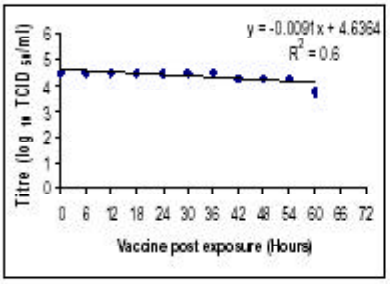
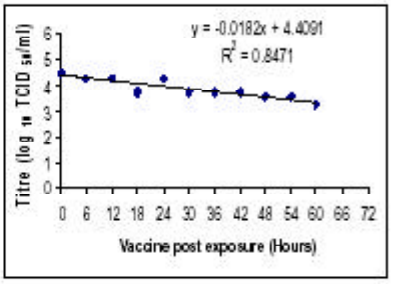


$MgSO_4$

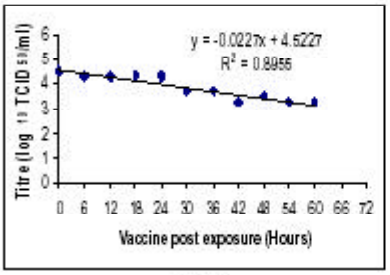
Fig 12. Degradation curves (45°C) for LS stabilized camelpox vaccine reconstitute with various diluents



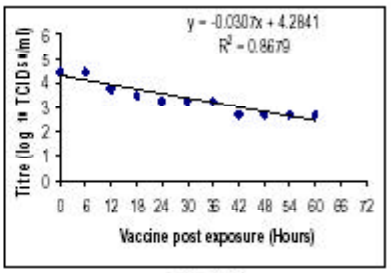
PBS



NaCl

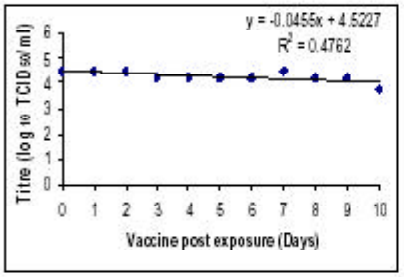


DW

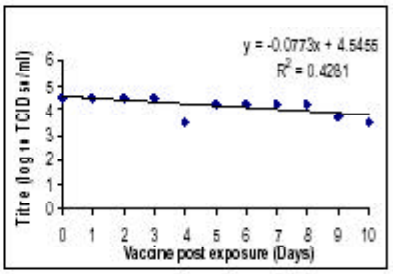


$MgSO_4$

Fig 13. Degradation curves (4°C) for BUGS stabilized camelpox vaccine reconstituted with various diluents



PBS



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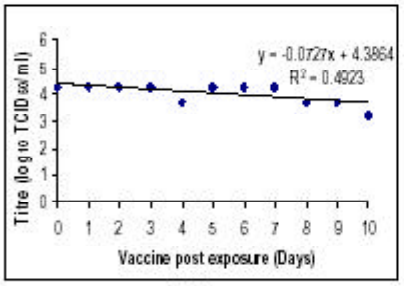

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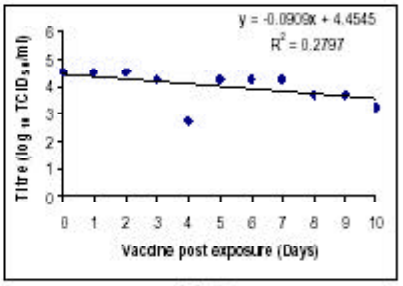
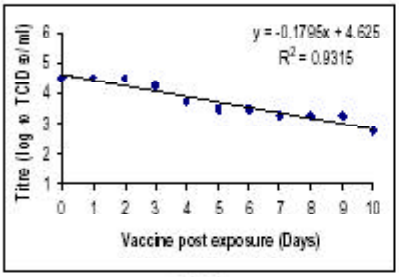


$MgSO_4$

Fig 14. Degradation curves (25°C) for BUGS stabilized camelpox vaccine reconstituted with various diluents



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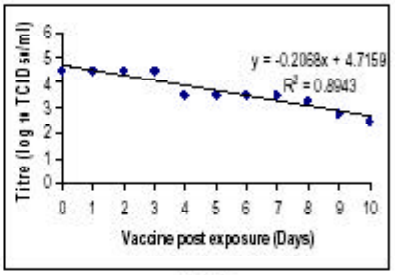

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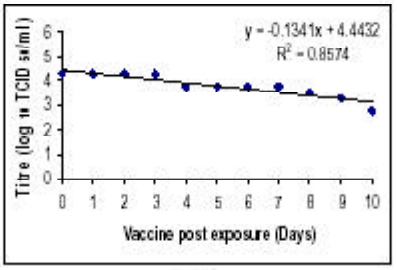

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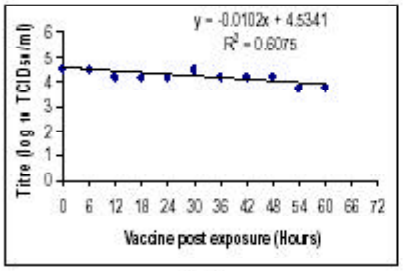
Fig 15. Degradation curves (37°C) for BUGS stabilized camelpox vaccine reconstituted with various diluents



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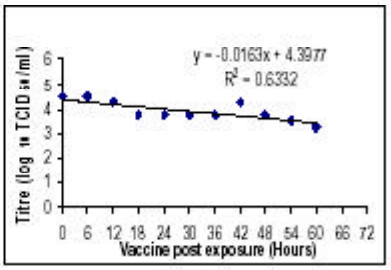

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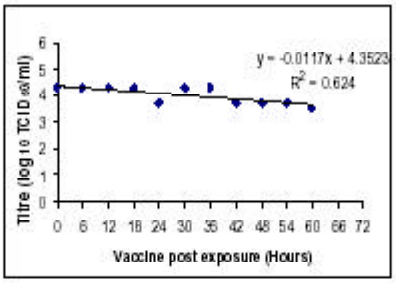

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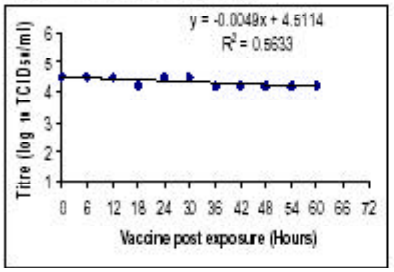
Fig 16. Degradation curves (45°C) for BUGS stabilized camelpox vaccine reconstituted with various diluents



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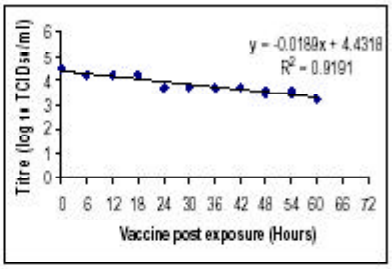

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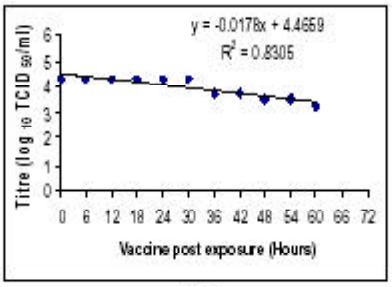


DW

Fig 17. Degradation curves (4°C) for TAA stabilized camelpox vaccine reconstituted with various diluents

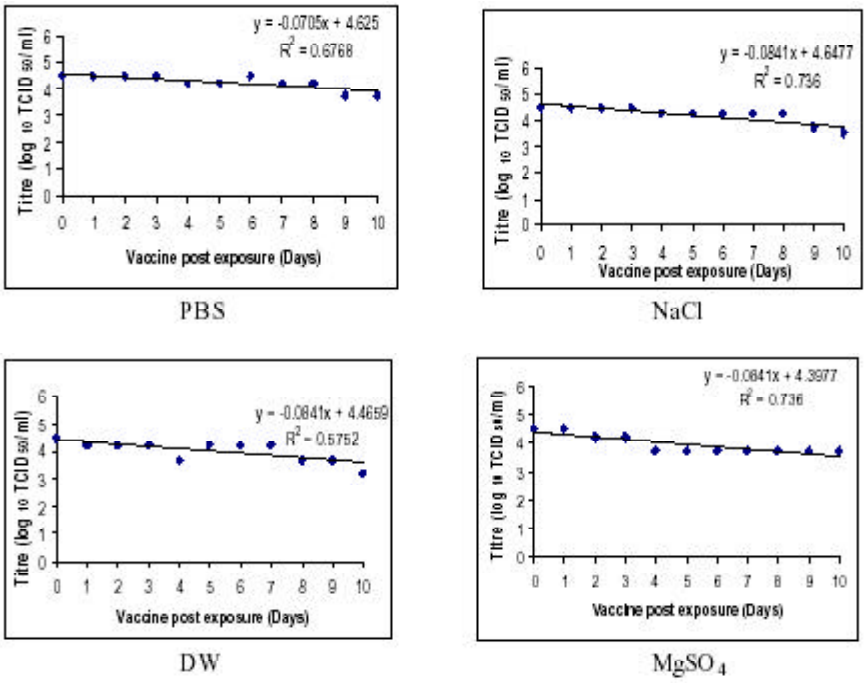


Fig 18. Degradation curves (25°C) for TAA stabilized camelpox vaccine reconstituted with various diluents

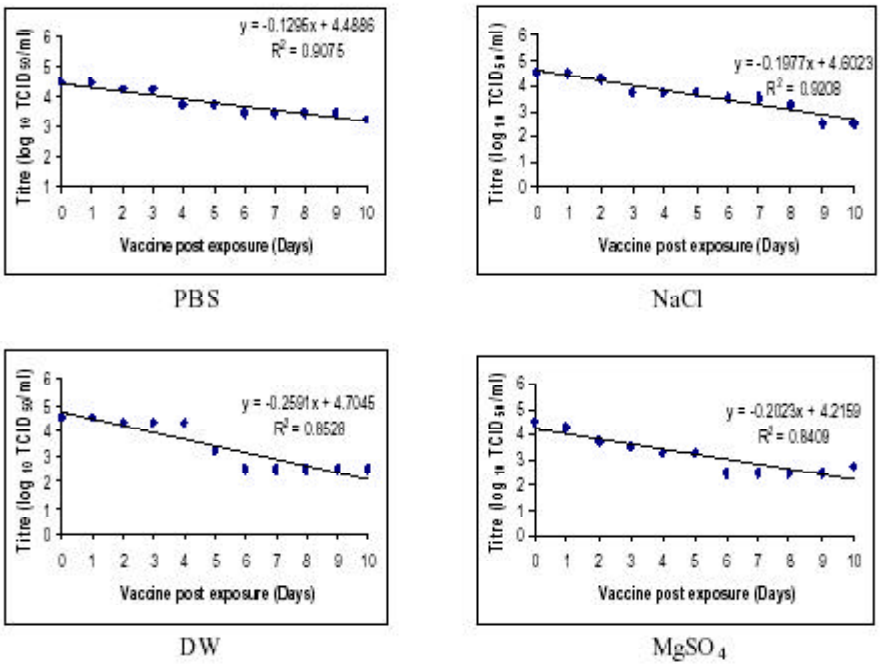
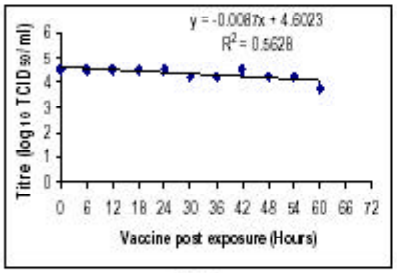


Fig 19. Degradation curves (37°C) for TAA stabilized camelpox vaccine reconstituted with various diluents



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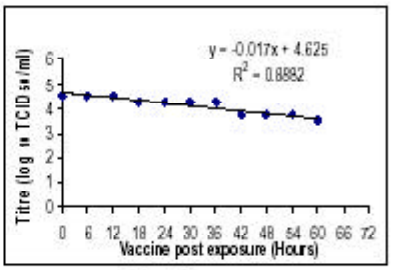

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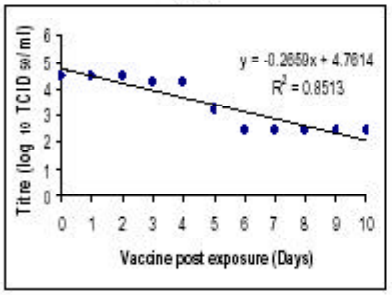

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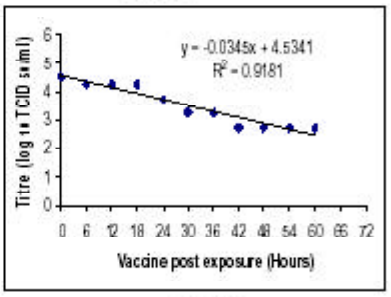
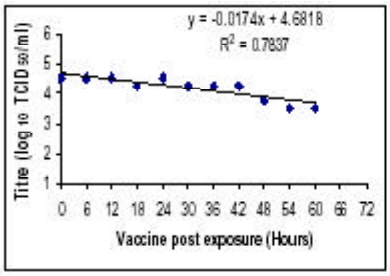


$MgSO_4$

Fig 20. Degradation curves (45°C) for TAA stabilized camelpox vaccine reconstituted with various diluents



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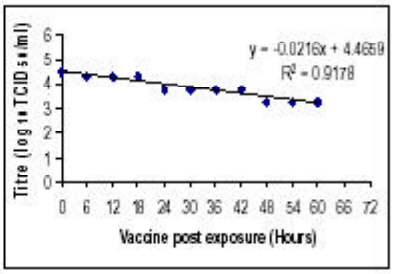

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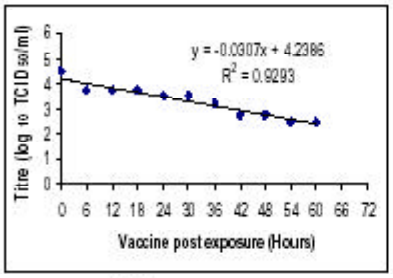

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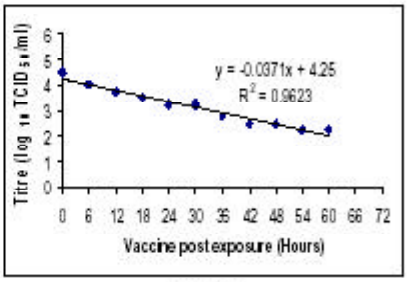

$MgSO_4$

based Q-PCR was found to be as less as 0.4 ng of DNA (Fig. 22). A total of eight different vaccine batches were employed for the study purpose to estimate the potency of attenuated live camelpox vaccine developed in our laboratory.

4.4.2. Establishment of standard reference vaccine

Freeze-dried (CMLV-1, P-50, batch 01/150409) live attenuated camelpox vaccine, which was produced in-house at Pox Virus Disease Laboratory, Division of Virology, IVRI, Mukteswar was selected for establishment of standard reference. The $TCID_{50}$ per ml of the reference was calculated by three consecutive titrations in Vero cell line and found to be $10^{6.56 \pm 0.09}$. The reference vaccine batch was stored at -20°C till further use. The titre of reference was re-checked at different time interval during storage to rule out any untoward effects of storage and found that there was no much change in the virus titre.

4.4.3. Establishment of critical time for harvest

The critical time to harvest vaccine batches was established by infecting the serially diluted CMLV-1 virus to 24 hour old confluent Vero cell monolayer in the six well tissue culture plates. The freeze-dried reference vaccine was reconstituted in EMEM medium and serially diluted ten-fold ranging from neat to 10^{-4}. Then 100 µl (0.1 m.o.i.) of each dilution was inoculated into six well plates with cell control. Each plate was harvested by freezing and thawing at 6 hr interval up to 42 hpi. The DNA was isolated from each harvest and subjected to Q-PCR to establish the standard curves. The experiment was repeated thrice and average slope of standard curves for each interval of harvest was compared. The maximum slope was obtained at 36 hpi. (-3.324) compared to other time harvests (Fig.23-24). Hence this time period was considered as critical time point for harvest of both the test and reference vaccine batches, to assign the comparative live virus titre for a given test batch of vaccine.

4.4.4. Validation of vaccine batches

A total of eight batches of live attenuated camelpox vaccine viz., 01/091209LS, 01/091209BUGS, 01/091209TAA, 02/161209LS, 02/161209BUGS, 02/161209TAA, 03/311209BUGS, 04/060110BUGS, and a reference batch of vaccine, 01/150409 were chosen for validation of Q-PCR. Later, 24 hr old confluent Vero cells were infected with ten-fold serial dilutions (neat to 10^{-4}) of all the test batches and reference. The DNA isolated from the

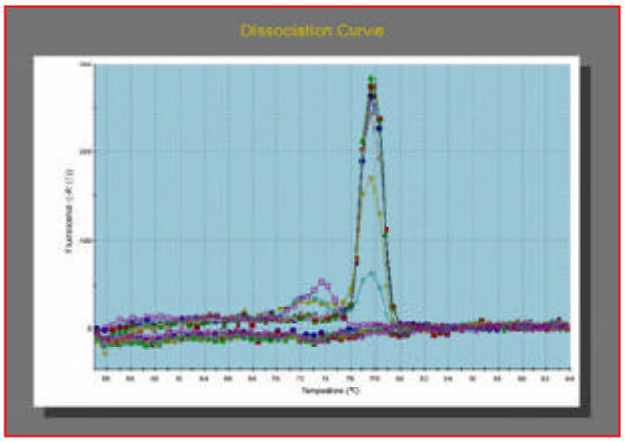

Fig. 21. SYBR Green dye based QPCR using CMLV C18L F and CMLV C18L R primer set showing the dissociation curves: Graphs shown in different colors represent purified CMLV DNA of dilutions ranging from 10^{-1} to 10^{-6}. BPXV DNA is represented by purple line at the bottom of the peak.

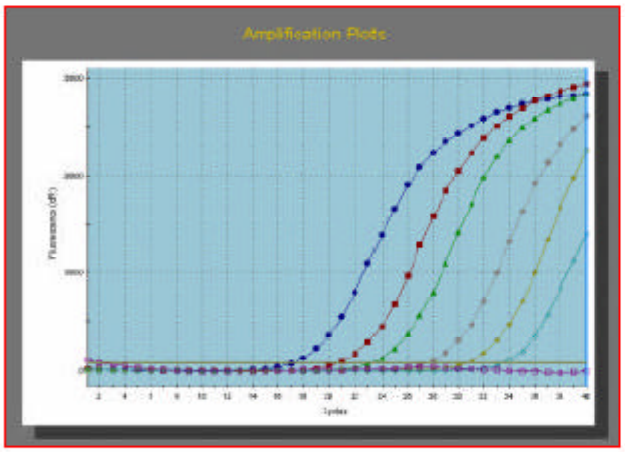

Fig. 22. SYBR Green dye based QPCR using CMLV C18L F and CMLV C18L R primer set for determining the sensitivity of the assay : Amplification plots corresponding to dilutions ranging from 10^{-1} to 10^{-6} of purified CMLV DNA and NTC. No amplification was noticed in the non-template control (purple).

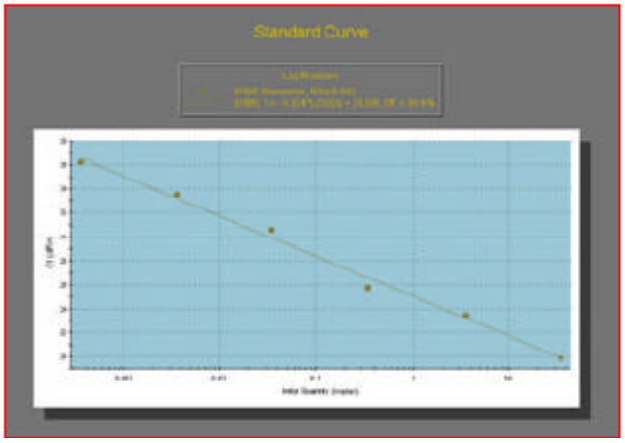

Fig. 23. SYBR Green dye based QPCR showing the standard curve : Using CMLV C18L F and CMLV C18LR, QPCR was carried out with the 10-fold serially diluted CMLV purified viral DNA. Each dot in the standard curve represents one of the dilutions of the purified CMLV viral DNA ranging from 10^{-1} to 10^{-6}.

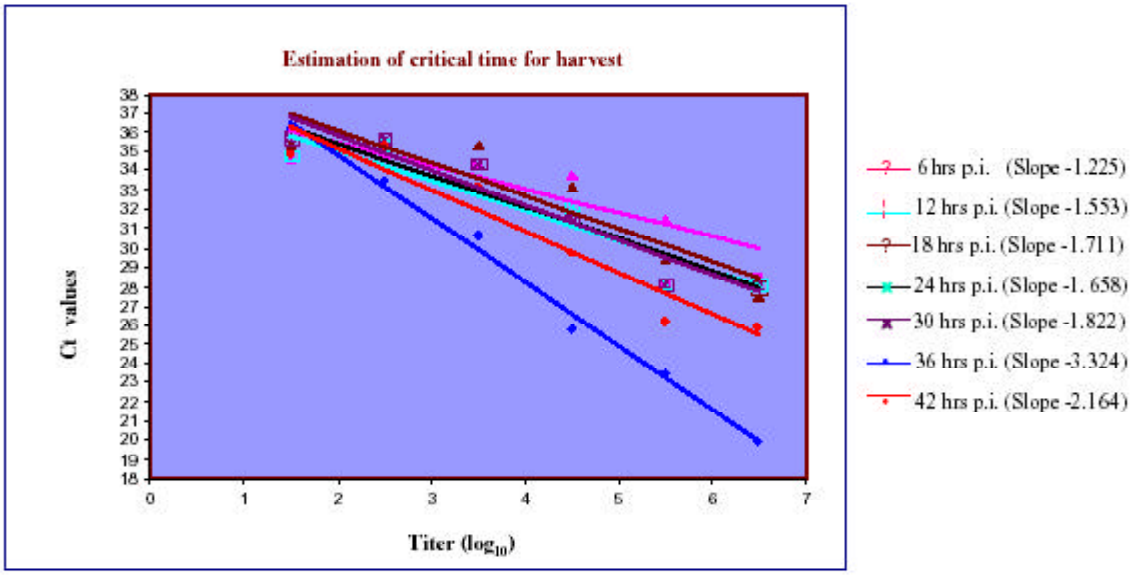

Fig.24. Standard curves established for different time interval harvest using SYBR Green based QPCR.

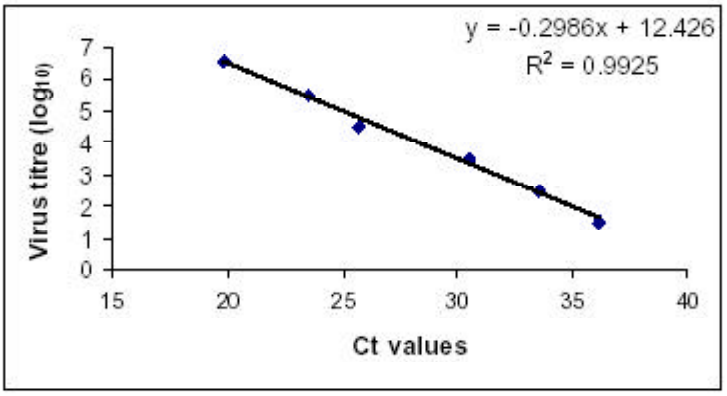

Fig.25. Standard curve showing linear relationship between the Ct values and $\log_{10}$ dilutions of the standard reference vaccine batch at 36 hpi.

Table 11. CMLV-I vaccine with LS stabilizer(Batch 01/091209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	23.03	5.57	6.77 ± 0.87	6.53±0.09
2	10^{-1}	23.78	6.30		
3	10^{-2}	25.25	6.81		
4	10^{-3}	26.63	7.60		
5	10^{-4}	29.28	7.61		

Table 12. CMLV-I vaccine with BUGS stabilizer(Batch 01/091209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	24.25	5.17	5.87 ± 0.58	6.23±0.09
2	10^{-1}	26.44	5.52		
3	10^{-2}	28.93	5.75		
4	10^{-3}	29.46	6.55		
5	10^{-4}	33.16	6.37		

Table 13. CMLV-I vaccine with TAA stabilizer(Batch 01/091209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	23.40	5.42	6.35 ± 0.73	6.53±0.09
2	10^{-1}	24.90	5.91		
3	10^{-2}	26.61	6.41		
4	10^{-3}	29.19	6.65		
5	10^{-4}	30.14	7.37		

Table 14. CMLV-I vaccine with LS stabilizer(Batch 02/161209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	20.11	6.45	7.30 ± 0.55	7.0±0.09
2	10^{-1}	21.33	7.05		
3	10^{-2}	22.07	7.85		
4	10^{-3}	26.00	7.61		
5	10^{-4}	29.53	7.55		

Table 15. CMLV-I vaccine with BUGS stabilizer(Batch 02/161209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	19.25	6.70	7.10 ± 0.39	6.67±0.09
2	10^{-1}	21.41	7.05		
3	10^{-2}	22.47	7.70		
4	10^{-3}	28.79	6.80		
5	10^{-4}	30.39	7.26		

Table 16. CMLV-I vaccine with TAA stabilizer(Batch 02/161209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	10^{-1}	20.49	6.33	7.17 ± 0.53	6.77±0.09
2	10^{-2}	21.61	6.97		
3	10^{-3}	23.41	7.43		
4	10^{-4}	26.20	7.58		
5	10^{-5}	29.48	7.55		

Table 17. CMLV-I vaccine with BUGS stabilizer(Batch 03/311209)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	10^{-1}	19.73	6.55	7.26 ± 0.49	6.5±0.09
2	10^{-2}	21.14	7.11		
3	10^{-3}	23.95	7.25		
4	10^{-4}	26.12	7.60		
5	10^{-5}	28.60	7.81		

Table 18. CMLV-I vaccine with BUGS stabilizer(Batch 04/060110)

Sl. No	Dilution	Ct Value	Corresponding titre/ml	Average titre/ml ± SD	$TCID_{50}$/ml
1	Neat	19.42	6.65	7.38 ± 0.52	6.5±0.09
2	10^{-1}	21.49	7.00		
3	10^{-2}	22.42	7.70		
4	10^{-3}	25.54	7.76		
5	10^{-4}	28.86	7.80		

harvests at 36 hpi was subjected to SYBR green based QPCR. Standard curve was established for the reference vaccine batch by plotting Ct values against log_{10} virus titres (Fig. 25). The Ct values of test vaccine batches were compared with the standard curve of the reference to determine the titre of the test vaccine (Table 11-18). The results obtained were compared with titre calculated in the Vero cell monolayer by conventional method and co-efficient of correlation was analyzed by student's t-test. The results of SYBR Green based Q-PCR were found to be comparable ($p>0.05$) without any significant difference and were within the acceptable limits.

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Chapter V

Discussion

Camelpox is one of the important infectious and contagious diseases diagnosed in the camelids. The disease was reported initially in Punjab and Rajaputana (India) (Leese, 1909) and later from many other countries like Middle East, Asia, African countries and some southern parts of Russia (Hafez *et al.*, 1992; Balamurugan *et al.*, 2008; Bhanuprakash *et al.*, 2010). It is confined to camel rearing belts particularly in developing countries and causes economic impact due to considerable loss in terms of morbidity, mortality, loss of weight and reduction in the milk yield.

Camelpox is caused by camelpox virus (CMLV), which belongs to the family *Poxviridae*, subfamily *Chordopoxvirinae*, genus *Orthopoxvirus* (ICTV, 2005). The disease is characterized by fever, enlarged lymph nodes and skin lesions (Wernery *et al.*, 1997a) and clinical manifestation range from inapparent and mild local infections confined to the skin, to moderate and less commonly, severe systemic infections. The camelpox virus was earlier thought as a zoonotic agent (Leese, 1909; Davies *et al.*, 1975) but so far a little evidence has been documented.

Routinely, camelpox is diagnosed based on clinical signs, epizootiological and pathological findings (Buchnev *et al.*, 1987), isolation of virus, electron microscopic detection of virus particles and genus-specific antigen capture enzyme linked immunosorbent assay. It could be differentiated from other orthopoxvirus (OPXV) and parapox infections by the use of restriction enzyme analysis (REA) (Murphy *et al*., 1999) and transmission electron microscopy (Al-Zi'abi *et al*., 2007). Recently, C18L gene based species specific PCRs (in conventional and real-time formats) have been developed to differentiate CMLV from BPXV and other OPXVs (Balamurugan *et al*., 2009).

Though the camelpox is economically important, literature on camelpox vaccine till 1992 was scantly. Earlier reports on camelpox vaccine were first published from the Soviet Union; however the details in respect of strain, safety and effectiveness were not available. Earlier a primitive method i.e. lacto-therapy had been followed in Punjab to control camelpox. Later only in 1973, there were reports of availability of camelpox vaccines from Russia, which were made from VACV rather than CMLV. Similarly, several reports of inactivated camelpox vaccines from Morocco (EL-Harrak *et al.*, 1991; 1998), Saudi Arabia (Hafez *et al.*, 1992) and the UAE (Wernery and Zachariah, 1999) have been reported later. These inactivated vaccines provide only 12 month protection and a large dose of vaccine is required to elicit protective antibody level. In the due course, attenuated virus strains were employed in Saudi Arabia and in the UAE for the production of live attenuated vaccine. The UAE attenuated CMLV vaccine (Ducapox®: Dubai camelpox vaccine) has been used since 1994 with a great success with more than six years protection. Subsequently, a Saudi isolate of CMLV designated as Jouf-78 was successfully employed for the production of live attenuated cell culture camelpox vaccine (Hafez *et al.*, 1992), manufactured commercially as "Orthovac®" and currently used in many countries.

Control of the disease becomes complex in countries where the disease is endemic and animal movement is difficult to restrict. To control any infectious disease, the basic requisite is the availability of a suitable vaccine. However in India, despite of economic importance and occurrence of sporadic cases of camelpox, so far attempts have not been made to produce vaccine. Furthermore, considering the disadvantages with inactivated vaccines viz. short duration of immunity and high antigenic mass required for vaccination, a live vaccine is the best choice as a long-term solution towards control of camel pox. With the right earnest, a Vero cell attenuated, live vaccine using local isolate of CMLV has recently been developed at the Division of Virology, IVRI, Muktesawar (Unpublished data). The virus isolate used for the vaccine is referred to as 'CMLV-1', which was isolated from the scab samples received from a suspected natural camelpox infection of camels in Bikaner, Rajasthan. The vaccine has undergone phase 1 (laboratory) and phase 2 (limited field) and is ready for phase 3 (large –scale field) trials. In the present study, the vaccine was evaluated for its potency in terms of number of live viral particle content in the vaccine based on a novel Q-PCR method and for thermo-stability (by

end point dilution method) which is one of the most important parameter before recommending the vaccine for field use.

Vaccine quality

Considering the field conditions of our country, this study was envisaged to compare the efficacy of different stabilizers in giving thermal protection to the candidate CMLV at various temperatures which are normally encountered at field conditions of subtropical countries like India. The vaccine formulations were made using the vaccine virus (CMLV-1) developed at IVRI, Mukteswar at a passage level of 50 which has been studied for its safety and efficacy (Unpublished data). A total of three stabilizer formulations were employed. These are (1) Lactalbumin hydrolysate (LAH)-sucrose (LS) reported by Mariner *et al.*(1990a) with modifications; (2) Buffered Gelatin and Sorbitol (Mariner *et al.,* 1990a) and (3) Trehalose with amino acids and divalent cations (TAA) reported by Adebayo *et al.*(1998). The vaccines with different aforementioned stabilizers were freeze-dried simultaneously using a conventional freeze-drying technique to compare the quality after lyophiliziation. Under these freeze-drying conditions, there was a loss of up to 0.75 $\log_{10}$ $TCID_{50}$ titre with respect to BUGS stabilizer. This loss has also been observed by Sarkar *et al*. (2003) who had used similar freeze-drying conditions. Although the BUGS stabilizer was prepared using the protocols described by Mariner *et al*. (1990a), it failed to prevent the loss during lyophilzation. These authors showed the initial loss of 0.5 $\log_{10}$ $TCID_{50}$ only, in case of TCRP vaccine. It is unlikely that this loss is due to lower pH of the BUGS stabilizer (pH=6.2) as CMLV was reported to be resistant to a pH range of 5-8.5 (Davies *et al.,* 1975). This loss may probably be prevented if the freeze-drying conditions described by Mariner *et al.* (1990a) are used.

Trehalose dihydrate, a non reducing disaccharide was used under conventional freeze-drying conditions with aminoacids (L-Alanine and L-Histidine) and divalent cations (Ca^{++} and Mg^{++} as salts). This stabilizer (TAA) showed a lyophilizaton loss of 0.08 to 0.50 $\log_{10}$ $TCID_{50}$ titre which is within the acceptable limits. The vaccine appeared granular and the mass was reduced compared to other stabilizers. The loss in titre during freeze-drying was also within the acceptable limit with the conventional LS stabilizer.

Residual moisture (RM) also contributes to the quality of any freeze-dried live vaccine (Precaustra *et al.,* 1982). In the present study, acceptable limit of RM was observed with

respect to BUGS stabilized vaccines. But, with the other two stabilizers, RM ranged between 3.28 to 4.54 %, which is considered to be relatively high. This high RM level may be due to the absence of heating phase of the vaccine vials during secondary drying.

Thermo-stability of the freeze-dried vaccine

Although thermostability studies of different live viral vaccines viz. RP vaccine (Plowright, 1963; Leiss and Plowright, 1963; DeBoer and Barber, 1964; Plowright *et al*., 1970, 1971; Bansal *et al*., 1974; Languet *et al*., 1985; Mariner *et al*., 1990a; Sreenivasa *et al*., 1997), PPR vaccine (Sarkar *et al*., 2003, Riyesh, 2008), has been exhaustively studied by several authors no such studies in respect to camelpox vaccines were conducted.

Additionally, 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 a best stabilizer to maintain recommended dose (5 $\log_{10}TCID_{50}$ per 100 doses vaccine vial) for a period as long as possible at a given temperature.

When the vaccines exposed at 4°C, all the three stabilizers were found to maintain the required infectivity titre during the entire study period of 5 months. Based on this limited duration of exposure, it revealed that the vaccine can be stored in the refrigerator for at least 5 months. However, vaccines with TAA stabilizer was found to be superior in terms of its shelf-life (44.11 months) and half-life (8.82 months) over other two stabilizers viz. LS (shelf-life - 34.88 months, half-life - 6.86 months) and BUGS (shelf-life - 13.44 months, half-life -2.75 months).

At 25°C, although both LS and TAA stabilizers had shown better stability compared to the other one and half-life wise, LS stabilizer was found to be superior (44.11 days) followed by BUGS (43.47 days) and TAA (40.54 days). If a freeze-dried vaccine contains an initial titre of around 6.53 $\log_{10}TCID_{50}$, it is expected to contain protective titre of 3 $\log_{10}TCID_{50}$ for 214-215 days when stabilized with LS or TAA. Whereas BUGS appears to be less effective stabilizer showing shelf-life of only 168.5 days.

At 37°C temperature, which is normally encountered in many parts of the country during summer months, vaccines with TAA stabilizer formulations had a relatively superior half-life (5.39 days) compared to 3.93 days for LS and 3.7 days for BUGS. At this temperature the vaccine having 6.53 $\log_{10}TCID_{50}$ infectivity titre stabilized with LS and TAA should be

used within 18 and 22 days, respectively. Here also BUGS appeared to have a shelf-life of 14.3 days only.

When vaccine formulations are exposed at 45°C, both half-life and shelf-life lasted for a couple of days only. Half-life ranged from 2.74 to 5.36 days, where as shelf-life ranged between 10.65 to 20.38 days. Of the three, TAA was found to be superior and the BUGS appeared less superior stabilizer for camelpox vaccine based on the 15 days study period.

The other factors which could be implicated in the poor performance of the vaccine at 37°C and 45°C may be due to the presence of relatively higher residual moisture (RM). Influence of RM on the viability of the vaccines has been reported earlier (Precausta *et al.,* 1982). Mariner *et al.* (1990a) and Sarkar *et al.* (2003) reported similar findings in case of TCRP and PPR vaccines, respectively. It has been suggested to extend the time period of secondary drying to ensure low RM as it was having detrimental effect on PPR vaccine (Worrall *et al.,* 2001). In studies conducted for TCRP (Mariner *et al.,* 1990a) and PPR (Worrall *et al.,* 2001) vaccines, it was reported that the maximum permissible RM should be below 2.5%. It has been showed that increasing the length and final temperature of lyophilization cycle will have a beneficial effect on the stability of the vaccine as it reduces the RM without affecting the infectivity titres. However in present 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 content of all the freeze-dried vaccine resulting in poor stability of the vaccine at higher temperatures of 37°C and 45°C.

Even though TCRP vaccines stabilized with BUGS was reported to have better stability (Mariner *et al.,* 1990a) like PPR vaccine (Sarkar *et al.,* 2003), BUGS stabilized camelpox vaccine was also not as stable as vaccines stabilized with LS or TAA. And it is difficult to explain that why BUGS failed to provide stability to camelpox vaccine. Whether acidic nature of BUGS (pH 6.2) had contributed to the downfall of stability can only be verified if a comparative study is done with BUGS stabilizer prepared at different pH range (pH 7.2).

It was suggested that increasing concentration of trehalose dihydrate may increase the stability of the vaccine (Worrall *et al.,* 2001). And it was reported that PPR vaccine stabilized

with trehalose dehydrate was as better as LS stabilizer under conventional freeze-drying conditions (Sarkar *et al.*, 2003; Riyesh, 2008). The current study was also supportive to this finding and indicating that TAA is a better substitute for camelpox vaccine. Overall, vaccines stabilized with TAA stabilizer showed better stability and the BUGS stabilizer was found to be a poor stabilizer for camelpox vaccines in the present study. However, as trehalose is very expensive, LS sabilizer can be used as alternative stabilizer for camelpox vaccine as it had shown almost comparable results as TAA stabilized vaccines.

Stability of the reconstituted vaccines

In the current study, the findings were quite interesting with the thermo-stability evaluation of reconstituted camelpox vaccine. Considering the field conditions, the diluted vaccines were kept under dry heat in transparent plastic tubes at 25, 37 and 45°C. The tubes were kept in refrigerator for 4°C.

Except with BUGS stabilized vaccine diluted with distilled water (0.25 $\log_{10}TCID_{50}$ loss in titre), immediate loss upon dilution was found to be nil with all of the vaccine formulations with different stabilizers diluted in various diluents viz. PBS, 0.85% NaCl, distilled water and 1M $MgSO_4$. It has also been found that in very few cases, the titre was slightly higher at 25°C than at 4°C. As the vaccines were reconstituted with chilled diluents and kept at 4°C it may be possible that the virus remained aggregated with cell debris at low temperature. When another aliquot of the same vaccine kept at 25°C, the virus could have disaggregated and resulted in higher titre. This may be studied in experimental animals, by inoculating the vaccines diluted with chilled diluents (probably aggregated form of virus) as well as after pre-warming at 25°C for at least one hour (virus in disaggregated form), as it would translate into different in antibody titre in animals. Further studies on pre-warmed diluents may throw light on this interesting result.

Earlier reports with other viral vaccines suggest that 0.85% NaCl and 1M $MgSO_4$ as choice of diluents especially with LS stabilizer (Anon, 1985; Mariner *et al.*, 1990a; Sarkar *et al.*, 2003). However, 1M $MgSO_4$ was not suitable for camelpox vaccine particularly with BUGS stabilizer in which it could not maintain the titre even for 6 hours at higher temperatures. The reason behind this was not ascertained. This may be due to the biological properties of

magnesium salts which depend on their interactions with water and with the polar groups at the membrane surface by screening and/or binding processes (Bara *et al.,* 1989). Except 1 M $MgSO_4$, the infectivity titre was more than the recommended dose (3 $\log_{10}TCID_{50}$), and titre was maintained even up to 60 hrs both at 37°C and 45°C with respect to other diluents. In the vaccines diluted with all four different diluents, there was no significant loss of infectivity titre over the sampling period of 10 days at 4°C. At 25°C, the vaccines could maintain the required titre ($10^3 \log_{10}TCID_{50}$) for the period of 5 days and thereafter, there was a reduction in infectivity titre with all diluents excluding PBS. Another interesting finding was that PBS maintained higher infectivity titre both at higher as well as at lower temperatures compared to other diluents with all vaccine-stabilizer formulations. Hence, our study confirms that PBS as diluent of choice for camelpox vaccine. These factors may be considered whenever the camelpox vaccination is launched in our country.

Potency estimation based on novel Q-PCR

Assigning the number of live virus particles per dose, is one of the important aspects of quality control as the efficacy of vaccine and seroconversion depends on number of live virus particles in attenuated live vaccines. Mostly potency assays of the live attenuated vaccines are done by plaque forming unit (pfu) assays and median cell culture infective dose assays (Schalk *et al.,* 2005). These conventional assays used for quantitation of infectious virus particles rely mainly on virus-induced cytopathology (Gaush and Smith, 1968). 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 titre in live attenuated vaccine. These assays are basically a subjective interpretation; hence there will be variation in the assigned titre between laboratory to laboratory, person to person and from time to time (Forsey *et al.,* 1992; 1993; Fukuda *et al.,* 1996). Thus, these assays suffer the disadvantages of labor intensity, time consumption, throughput restriction and variability. These traditional assays are not the preferred tests of choice to assign titre to the individual components of multivalent or bivalent 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; 2005; Kallesh *et al.*, 2009). This assay 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 (Cycle threshold, 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 and does not correspond to infectious load. To overcome this drawback, cell cultures were infected with vaccine sample and harvested at critical time point for QPCR. As only live virus particle 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 (Schalk *et al.*, 2004), rotavirus in pentavalent rota virus vaccine, Rota teq® (Ranheim *et al*., 2006), multivalent measles, mumps and rubella vaccine (Schalk *et al*., 2004; 2005) and to assign infectious potencies to adeno virus based vaccine and vectors for gene therapy (Wang *et al.*, 2005).

One of the most important advantages of QPCR based estimation of titre is that the titre 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 CMLV, the CPE appears on 24 hpi and completed before 3 day p.i. In the current study, QPCR is applied to determine the titre of CMLV in a given batch of vaccine as early as 36 hpi which drastically reduced the time and labor required. At the same time this assay is technically simple, specific, sensitive, reproducible and applicable to estimate the titre of the individual components in multivalent or bivalent or combined vaccines. Additionally, results obtained are comparable to the convention endpoint dilution methods of titre estimation.

In the present study, the infectivity titre of standard reference vaccine virus was found to be $10^{6.5 \pm 0.09}$ $TCID_{50}$ /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 for cells post infection to estimate the live viral particles in given test vaccine batch was determined. The critical time point for harvest corresponds to the minimum time required for appearance of sufficient viral particles in all the dilutions of the virus infected. The SYBR Green based QPCR was carried out with the samples harvested at 6, 12, 18, 24, 30, 36, and 42 hpi The experiment was repeated thrice and average slope of standard curves for each interval of harvest was compared. The maximum slope was obtained at 36 hpi (-3.324) compared to other time harvests. 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, a total of eight batches of live attenuated camelpox vaccine were selected to estimate the titre. The results obtained were found to be satisfactory and with in the acceptable range of accuracy. There was a moderate degree of correlation (0.6633) was observed between the tires estimated by the QPCR and conventional infectivity assay with no significant difference ($p>0.05$) noticed between the methods. These findings are in agreement with earlier findings (Kallesh *et al.*, 2009).

Conclusions

The findings with respect of thermo stability of camelpox vaccine revealed that the vaccine was quite stable at ambient temperature irrespective of the stabilizer. The stabilizer formulation such as trehalose dihydate with amino acids and divalent cations (TAA) followed by lactalbumin hydrolysate-sucrose (LS) was appeared superior to BUGS stabilizer studied. As economics is the major constraint in most of the developing countries LS stabilizer can also be used as alternative to TAA for camelpox vaccine. Using the vaccine virus culturing techniques and conventional freeze-drying conditions described in this study, the camelpox vaccine stabilized with TAA or LS will have an expiry period of 35-44 months at 4°C, 214-215 days at 25°C, 18-22 days at 37°C and 16-20 days at 45°C. The refinement in freeze-drying technique is

suggested to increase the expiry period of the vaccine in lyophilized form. Based on this study, it is now possible to prescribe a time limit for use of reconstituted vaccine. And for reconstitution, phosphate buffer saline (PBS) is a diluent choice followed by normal saline solution (0.85% NaCl), but 1M MgSO4 is less suitable for camelpox vaccine. The reconstituted vaccine can be safely used for more than 60 hrs even at higher temperatures of 37°C and 45°C.

The SYBR Green based QPCR was successfully employed to estimate the titre of camelpox virus in attenuated live camelpox vaccine and the critical time point for harvest was determined as 36 hpi to estimate the maximal virus load. The titres obtained in this novel QPCR assay and the conventional end point dilution methods ($TCID_{50}$) were quite comparable with each other without any significant difference ($p>0.05$) in less time period.

Further, this study also leads to following new areas that should be explored. These areas may be:-

- To continue the stability studies of both freeze-dried as well as reconstituted camelpox vaccine beyond this study period.
- To study the thermo stability of camelpox vaccine stabilized with BUGS prepared at pH 7.2
- To study the intrinsic stability of camelpox vaccine virus at different temperatures for different time periods
- To study the difference in antibody titre between the animals inoculated with vaccines reconstituted with chilled and pre-warmed diluents.
- To observe the effects of 1M MgSO4 on the camelpox vaccine virus at molecular level especially at higher temperatures.
- To continue further validation of this quick as well as novel QPCR method of potency estimation to check the repeatability and reproducibility for its wider application.

Chapter VI

Summary

Camelpox is the contagious skin disease of camelids caused by camelpox virus (CMLV) and characterized by mild local skin infection and less common severe systemic infections. The disease is confined to camel rearing belts of developing countries and causes economic impact due to considerable loss in terms of morbidity, mortality, loss of weight and reduced milk yield. Both inactivated and live attenuated vaccines are available in few countries. However in India, despite of economic importance and occurrence of sporadic cases, so far attempts have not been made to develop camelpox vaccine. Furthermore, considering the disadvantages with inactivated vaccine viz. short duration of immunity and high antigenic mass required for vaccination, a live vaccine is the best choice as a long-term solution towards control of camelpox. With the right earnest, a Vero cell attenuated live vaccine from an Indian isolate of CMLV (named CMLV-1) has been developed recently at IVRI, Mukteswar. The vaccine has undergone laboratory trials for sterility, innocuity, safety, potency and efficacy. But a study with respect to thermo stability has not been undertaken. In the present study, the vaccine was evaluated for its potency based on a Q-PCR method and for thermostability which is one of the most important parameters before recommending the vaccine for field use.

Three stabilizer formulations were employed to test the thermo stability of the vaccine. Two stabilizer combinations viz. Lactalbumin hydrolysate-sucrose (LS) and Buffered Gelatin and Sorbitol (BUGS) have been studied extensively on TCRP and PPR vaccines. The other one was modern stabilizer [Trehalose with amino acids and divalent cations (TAA)] reported for RP, PPR and yellow fever virus vaccines. The vaccines with aforementioned stabilizers were freeze-dried simultaneously under similar freeze-drying conditions to compare the quality of the vaccine and to know the loss of titre during lyophilization. Further, the residual moisture

(RM) for each batch was calculated by thermo gravimetric method, as it has impact on the viability of lyophilized vaccine.

Thermo-stability study of the camelpox vaccine was carried out using both freeze-dried as well as reconstituted forms of the camelpox vaccine. The freeze dried vaccine vials were exposed to four different temperatures (4, 25, 37 and 45°C) for different time periods and titrated to know the loss of infectivity titre. In reconstituted form, four diluents (PBS, 0.85% NaCl, distilled water and 1M $MgSO_4$) were used. Each vaccine vial (100 doses) containing different stabilizers were reconstituted with 100 ml of each diluent and titrated immediately to know the loss upon dilution. Then the diluted vaccine was divided in aliquots and exposed at 4, 25, 37 and 45°C. The samples were taken 6 hourly for 4°C and 25°C up to 60 hrs and daily for 37°C and 45°C up to 10 days and titrated. The data obtained were analyzed by regression analysis.

In the current study, the loss of titre during lyophilization was found to be lowest for TAA and highest for BUGS stabilizers. The acceptable limit of RM was observed with respect to BUGS stabilizer than other two which had shown relatively high RM than the desired level. At 4°C, although TAA stabilizer was found relatively superior in terms of both shelf-life (44.11 months) and half-life (8.82 months), the other two were also good enough in maintaining the titre. At 25°C, shelf-life of both LS and TAA stabilizers was found to be 214 days. However, half-life was longest for LS stabilizer (44.11 days) followed by BUGS (43.47 days) and TAA (40.54 days). At 37°C, TAA stabilizer formulations had a relatively superior half-life (5.39 days) compared to 3.93 days for LS and 3.7 days for BUGS. When exposed at 45°C, both half-life and shelf-life lasted only for a couple of days with all stabilizers, ranging from 2.74 to 5.36 days, where as shelf-life ranged between 10.65 to 20.38 days. At all temperatures, BUGS stabilizer appeared less effective compared to others.

The stability of reconstituted vaccine was quite encouraging. Except BUGS stabilized vaccine diluted in distilled water, there was no loss on dilution for all vaccine stabilizer formulations diluted in various diluents. In the vaccines diluted with all diluents, there was no significant loss of infectivity titer over the sampling period at 4°C. The titer was slightly higher at 25°C than at 4°C for LS stabilized vaccine diluted in 0.85% NaCl and for vaccine with TAA stabilizer diluted with distilled water. This may be due to the fact that the virus may remain in aggregated

form when reconstituted with chilled diluent and keeping subsequently at 4°C. Although there was marginal decrease, the infectivity titre was more than the recommended dose (3 $log_{10}TCID_{50}$) and maintained even up to 60 hrs both at 37°C and 45°C by all diluents except 1 M $MgSO_4$. This study also suggests that PBS as diluent of choice for camelpox vaccine as it could maintain higher infectivity titer both at higher as well as lower temperature.

Apart from stability, assigning the number of live virus particles per dose is one of the important aspects of quality control as the efficacy of vaccine and seroconversion depends on number of live virus particles in attenuated live vaccines. Conventional assays used for quantitation of infectious virus particles rely mainly on virus-induced cytopathic effects. These are basically a subjective interpretation, and there will be variation in the assigned titer between laboratory to laboratory, person to person and from time to time. Even though these assays are considered to be the gold standard, certain drawbacks as mentioned previously precludes them from long-term implementation for estimation of titer in live attenuated vaccine. Recently, QPCR has been used as a novel approach for the potency estimation of vaccines due to high sensitivity, specificity, reproducibility and rapidity.

In the current study, the estimation of live camelpox virus particles in the attenuated vaccine using QPCR as an alternative approach to conventional endpoint dilution method was investigated. One of the camelpox vaccine batches was selected as standard reference and titre of which was found to be $10^{6.5+0.09}$ $TCID_{50}$ /ml based on three consecutive titrations. The ten-fold serial dilutions (neat to 10^{-4}) of reference vaccine virus was infected to 24 hrs old confluent Vero cell monolayer and harvested at 6, 12, 18, 24, 30, 36, and 42 hrs post infection (hpi). The DNA isolated from all intervals of harvest was subjected to the SYBR Green dye based QPCR. The standard curves were established by plotting Ct values obtained from three consecutive experiments against log titre of dilutions. Of which, the 36 hrs of harvest with the highest slope (-3.324) was selected as critical time to harvest the reference as well as test vaccine batches.

For validation of QPCR assay, a total of eight different batches of live attenuated camelpox vaccine were selected. These were infected to cells, harvested at 36 hpi and all the isolated DNA were subjected to QPCR. 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 quite comparable to each other as analyzed by student's t-test. Correlation co-efficient of the titres in potency estimation of vaccine batches by SYBR Green dye based QPCR was within the acceptable limit. Hence, the QPCR assay could be explored for its potential to replace or an adjunct to conventional method of end point titration for assaying the live camelpox virus load in a given batch of vaccine. Nevertheless, further validation of this quick as well as novel QPCR method of potency estimation is warranted to attribute repeatability and reproducibility for its widespread application.

Chapter VII

Mini Abstract

Camelpox is the contagious skin disease of camelids caused by camelpox virus (CMLV). The disease causes economic impact in terms of morbidity, mortality, loss of weight and reduced milk yield. Although, vaccines are available to control the disease in few countries, in India so far attempts have not been made to produce camelpox vaccine. Recently, an attenuated live virus vaccine has been developed at IVRI, Mukteswar and it was found safe, potent and efficacious. The current study deals with evaluation of potency and thermostability of the vaccine virus. In thermo-stability study, a total of three stabilizers were used for freeze-drying the vaccine. Stability of both freeze-dried and reconstituted vaccine was tested at different temperatures. Based on various time periods studied, it was revealed that the camelpox vaccine lyophilized with TAA stabilizer appeared relatively superior with an expiry period of 44 months at 4°C, 215 days at 25°C, 22 days at 37°C and 20 days at 45°C. On the basis of half-life also, TAA appeared better followed by LS and BUGS at all temperatures except at 25°C in which LS found relatively superior. Among the three stabilizers, BUGS stabilizer appeared relatively inferior to others at all temperatures. Four diluents viz. PBS, 0.85% NaCl, distilled water and 1M $MgSO_4$ were used for reconstitution of the vaccine. Of which, PBS appeared better for reconstitution followed by 0.85% NaCl and both the diluents maintained the infectivity titre more than the recommended dose (3 $\log_{10} TCID_{50}$) up to 60 hrs both at 37°C and 45°C. Among the diluents tested, 1M $MgSO_4$ was less suitable for camelpox vaccine. For potency estimation, rapid and novel SYBR Green based QPCR was successfully employed to estimate the titre of CMLV in attenuated live camelpox vaccine and the critical time point for harvest was determined as 36 hpi. To validate the assay, eight different test batches of vaccines were selected and QPCR was carried out using DNA isolated from harvests at 36 hpi with various dilutions of test batches of vaccine viruses. The titre of test batches was calculated by comparison of its Ct values with that of standard curve of the reference. The titers estimated by QPCR and $TCID_{50}$ methods were well comparable to each other as analyzed by student's t-test without any significant difference ($P>0.05$). Thus the assay could be used as a quick alternative for estimation of live CMLV particles in the vaccine. From the studies conducted, the vaccine was found relatively stable at different temperatures and the QPCR was suitable for estimating the titre of the vaccine.

Chapter VIII

लघु सारांश

ऊँट चेचक ऊँटो का एक संक्रामक त्वचा रोग है जो ऊँट चेचक विषाणु द्वारा होता है। यह रोग आर्थिक रूप से अस्वस्था, मृत्यु, वजन में कमी और दुग्ध उत्पादन में कमी करता है। यद्यपि कई देशों में रोग कि रोकथाम के लिए वैक्सीन उपलब्ध है, पर भारत में अब तक ऊँट चेचक वैक्सीन उत्पादन के लिए कोई कोशिश नहीं की गई। हाल में एक तनुकृत विषाणु वैक्सीन आई.पी.आर.आई. मुक्तेश्वर में बनाया गया और ये सुरक्षित, प्रबल और समर्थ पाया गया। वर्तमान अध्ययनइस टीके के विषाणु की प्रबलता और ताप सहन क्षमता के अध्ययन से संबंधित है। ताप सहन क्षमता अध्ययन के लिए कुल तीन स्थितीकरण पदार्थो का उपयोग टीके के फ्रीज ड्राईग के लिए किया गया। फ्रीज ड्राईड और पुर्णगठित दोनो टीके की सहन क्षमता विभिन्न तापमानों पर आंकी गई। विभिन्न समय अवधि के आधार पर ऊँट चेचक टीका, टी.ए.ए. स्थिरता के साथ लायोकिलाइजड किया गया, की अवसान अवधि 44 महीने 4^0 सेल्सीयस पर 215 दिन 25^0 से. पर 22 दिन 37^0 से. पर और 20 दिन 45^0 से. पर उतम पायी गयी। अर्द्ध जीवन के आधार पर भी, टी.ए.ए. उपयुक्त पाया गया, तत्पश्चात एल.एस. और बगस सारे तापमानों पर उपयुक्त पाये गये सिर्फ 25^0 सेल्सियस पर जहाँ एल.एस. उत्तम पाया गया। चार तनुकारक पी.बी.एस., 0.85 प्रतिशत सोडियम कलोराइड, आस्रुत जल और 1 आणुविक मैग्निशियम सल्फेट टीके के पुर्नगठन में प्रयुक्त किये गए। जिसमें से पी.बी.एस. बेहतर तनुकारक पाया गया तत्पश्चात 0.85 प्रतिशत सोडियम कलोराइड और दोनो तनुकारक संक्रमित टाइटर योग्य खुराक से ज्यादा ($3 \log 10\ TCID_{50}$) 37^0 से. और 45^0 से. पर 60 घण्टे तक अवलम्बन रखते हैं। तनुकार को जो आंके गए में से, 1 आण्विक मैग्निशियम सल्फेट चेचक विषाणु टीके के लिए कम उपयुक्त पाया गया। प्रबलता के लिए शीघ्र और अपूर्व साईबर ग्रीन पर आधारित क्यू.पी.सी.आर., तनुकृत ऊँट चेचक टीके में सी.एम.एल.बी. की टाइटर आंकने के लिए नियुक्त किया गया। और टीके विषाणु के कई तनुकारकों के साथ महत्वपूर्ण समय 36 घण्टे संक्रमण पश्चात आंका गया। जांच समूह का टाइटर इसके Ct मूल्य के साथ संर्दभ के सिद्धांत कर्व की तुलना का आंकलन द्वारा किया गया। क्यू.पी.सी.आर. और टी.सी.आई.डी. 50 द्वारा आंका गया टाईटर एक दूसरे के तुल्नात्मक था जैसा कि विद्यार्थी टी–टेस्ट द्वारा बिना किसी महत्वपूर्ण विभन्नता ($P<0.05$) मापा गया। इसलिए यह परीक्षण जीव सी.एम.एल.वी. टीके मापने के लिए एक तेज विकल्प के रूप में प्रयोग हो सकता है। अध्ययन से टीका विभिन्न तापमानों पर स्थिर पाया गया और क्यू.पी.सी.आर. टीके की टाइटर मापने के लिए उपयुक्त है।

Chapter IX

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Appendix

A. Cell culture media and buffers

Eagle's Minimal Essential Medium (EMEM) (1 Liter)

EMEM powder (Sigma)	9.4 g/L
$NaHCO_3$ (7.5%)	15 ml/L
L-Glutamine (3.0%)	0.292 g/L 10 ml
Penicillin	100 IU/ml
Streptomycin	100 mg/ml

Dissolve the EMEM powder in 1 liter of distilled water and autoclave at 120ºC at 15 lb pressure for 15 minutes. Keep at 4ºC till use. Glutamine, $NaHCO_3$ and antibiotic solutions were added in media just before use.

Trypsin-Versene (1000 ml)

Versene	0.700 gm
D-Glucose	1.000 gm
KCl	0.400 gm
NaCl	8.000 gm
$NaHCO_3$	0.580 gm
Trypsin	1.000 gm
Phenol red	0.002 gm
Distilled water	up to 1000 ml

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

Hank's Balanced Salt Solution (HBSS)

NaCl	8.000 gm
KCl	0.400 gm
KH_2PO_4	0.060 gm
$Na_2HPO_4 .2H_2O$	0.057 gm
Phenol red	0.011 gm
DW	up to 1000 ml

pH adjusted to 7.4 and sterilized by autoclaving at 121°C at 15 lb for 15 minutes.

1X Phosphate Buffered Saline (pH 7.4)

NaCl	8.000 gm
KCl	0.200 gm
KH_2PO_4	0.200 gm
$Na_2HPO_4.2H_2O$	1.420 gm
DW	up to 1000 ml

Soya casein digest media

Soya casein digest media powder	3 gm
Distilled water	100 ml

Dissolved the contents properly in distilled water and autoclaved at 121ºC at 15 lb pressure for 15 minutes

B. 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 50^0C 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 $MgCl_2$	1.0 ml
Yeast Extract	0.5 gm
Distilled Water	up to 100 ml

SOC Medium (100 ml)

SOB Medium	98 ml
1M Glucose	2 ml

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

X-gal	20 mg
N'N' Dimethyl formamide	1 ml

Stored at -20^{0}C

IPTG

IPTG	20 mg
Distilled water	1 ml

Sterilized by filtration

Ampicillin

Ampicillin	100 mg
Distilled water	1 ml

Dissolved the contents properly and filtered through 0.45µm filter

C. Solutions for plasmid isolation

Solution-MXI

100X TE buffer (Sigma)	0.5ml
2M Glucose	1.25ml
Distilled Water	up to 50 ml

Solution-MXII

10% SDS	5 ml
10M NaOH	0.5ml
Distilled Water	up to 50 ml

Solution-MXIII (pH 4.8)

5M Potassium Acetate	100ml
Glacial Acetic Acid	19.6 ml
Deionized Water	47.50ml

The solutions were then filtered and autoclaved.

5M Potassium Acetate

Potassium Acetate	49.0 gm
Distilled Water	up to 100 ml

5M Ammonium Acetate

Ammonium Acetate	38.5gm
Distilled Water	up to 100 ml

The solution was then filtered

5M NaCl

NaCl	29.22 gm
Distilled Water	up to 100 ml

The solution was then autoclaved.

Poly ethylene glycol (8000) with 60mM Mg^{++}

PEG (8000)	30 mg
Distilled Water	1000 ml

Autoclave and add 2M Mg^{++} (3 ml) solution afterwards.

TE Buffer

Tris HCL (pH 8.0)	10 mM
EDTA	1 mM

10 mM dNTP mix

dATP	10 mM
dCTP	10 mM
dGTP	10 mM
dTTP	10 mM

Loading buffer (6X)

Bromophenol blue	0.25% w/v
Xylene cyanol FF	0.25% w/v
Sucrose in water	40% w/v

10X TAE (Tris Acetate EDTA) Buffer, pH 8.8

Tris Base	48.2 gm
Glacial acetic acid	11.4 ml
EDTA (0.5M, pH 8)	10.0 ml

Add distilled water to make final volume 1000 ml.

Ethidium bromide

Ethidium bromide	10 mg
Distilled water	1 ml

Mixed and stored in a dark place

Vitae

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