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Cell culture adapted sheeppox virus as a challenge virus for potency testing of sheeppox vaccine

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Sheeppox virus from an outbreak of sheeppox that occurred in Srinagar (Jammu and Kashmir, India) in 2000 was isolated by inoculation of susceptible sheep and further re-isolated in cell culture. The field virus, adapted to grow in lamb testes culture, was evaluated for its potential use as challenge virus in potency testing of sheeppox vaccine currently in use. The virus (passage 6) produced severe disease in susceptible sheep when inoculated subcutaneously with a dose of $10^{6.2}$ $TCID_{50}$. The virus identity was confirmed by PCR, sequencing of P32 gene and species-specific signature residues identified in deduced aa sequence of the gene. The virus was successfully evaluated for its virulence using two batches of sheep pox vaccines. Use of this field virus enables consistent potency experiments of sheeppox vaccines avoiding use of animals for its propagation and titration.

Keywords: Cell culture, Potency testing, Sheeppox vaccine, Sheeppox virus, Virulent virus challenge.

Sheeppox is a notifiable disease of Office International des Epizooties (OIE). The disease is recognized as most fatal in sheep, characterized by formation of dermal pox lesions and nodular lesions on the respiratory and digestive systems. The disease causes high morbidity (100%) and mortality up to 75% in young and immunologically naïve animals. The causative sheeppox virus (SPPV) is classified in the genus *Capripoxvirus* along with goatpox virus (GTPV) and lumpy skin disease (LSDV) of *Poxviridae* family[1]. These viruses are closely related antigenically[2,3]. A number of outbreaks of sheeppox have been reported in the recent past[4-7]. The live attenuated sheep pox vaccine is used for mass immunization as a preventive measure in India. Hence, a large quantity of vaccine needs to be produced by many public and privately owned vaccine-manufacturing units to meet the national requirement. Potency testing of live attenuated sheep pox vaccine was performed in sheep using scab suspension derived from animals experimentally infected with virulent SPPV with a minimum dose of skin reactive $dose_{50}$ ($SRID_{50}$)$10^{5.0}$/ml per animal where the exact number of viral particles cannot be ascertained.

In the present study, a field isolate of SPPV from Srinagar district of Jammu and Kashmir, India was isolated and characterized for its potential use as a challenge virus in potency studies. The virus was initially revived in sheep and subsequently adapted in Primary lamb tests (PLT) cell culture. The virus grown in bulk at 6th passage level was tested for producing experimental infection in a susceptible sheep. A major immunogenic gene (P32) of the virus was also amplified by PCR and its sequence determined to confirm the virus identity.

Materials and Methods

Virus isolation—Scab suspension (10%w/v) from the outbreak was used for experimental reproduction of the disease in a sheep. The virus is maintained in the laboratory as a scab form through regular animal passages. It was isolated in primary/secondary lamb testes (SLT) cell culture grown in Glasgow's modified minimum essential medium (GMEM, Sigma, St Louis, USA) supplemented with 10% newborn calf serum (HyClone, USA). For virus isolation, sonicated scab suspension was added on to LT cell monolayer and allowed for viral adsorption for 1 hr, in 1ml of GMEM. Un-adsorbed virus was then removed by washing the monolayer thrice with GMEM. Cells were fed with medium containing 2% serum and observed daily for appearance of specific cytopathic effects (CPE).

PCR and sequencing—Viral DNA was extracted from cell culture virus using AuPreP DNA Extraction

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Kit [M/s Life Technologies (India) Pvt Ltd, New Delhi, India] as per manufacturer's protocol. Diagnostic PCR was carried out using primers-B68 and B69[8]. Full-length P32 gene was also amplified using the A95 and B7 primers[8]. PCR amplicon was purified using MinElute gel extraction kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions, ligated to pGEMT-Easy vector (Promega, USA) and transformed into competent *E.coli* strain (TOP10, Invitrogen, USA). Sequencing of the PCR amplicon was carried out using an automated DNA sequencer (ABI PRISM 3100, Perkin Elmer) in either direction.

Sequence analysis—Nucleotide and deduced amino acid sequences were compared with other representative capripoxvirus isolates from different regions and sequence identity (%) was determined using Clustal W method[9].

Animal inoculation and virus confirmation—Virus titrated[10] in SLT culture was inoculated into susceptible sheep. Animal was inoculated subcutaneously with $10^{6.2}$ $TCID_{50}$ of virus (passage 6) in 1.0 ml dose at multiple sites @ 0.1 ml/site. Animal was observed daily for rectal temperature and appearance of systemic or local responses. After experimental infection, virus was confirmed by detection of viral nucleic acid in the scab samples collected from the affected animals using PCR[8].

Validation of the virus for challenge studies—Two batches of sheep pox vaccines, made using Rumanian Fanar from Veterinary Biologicals, Lucknow and Srinagar strain from the Division of Virology were tested using this challenge virus as per the prescribed protocol[11]. Two groups each of 6 sheep, which were sero-negative to SPPV antibodies, were administered with a recommended field dose ($10^{2.5}$ $TCID_{50}$ in 0.2 ml) of each vaccine at the abaxial surface of the tail. A third group of two sheep was maintained as in-contact control. Temperature of all the animals was recorded for a period of 14 days following vaccination. Appearance of clinical symptoms was also observed regularly. Then, all the animals were challenged with virulent SPPV (Srinagar 38/00 isolate, 6th passage) at $10^{6.5}$ $TCID_{50}$/animal intradermally at lower abdomen on day 28 post immunization. The appearance of clinical symptoms and temperature response were recorded daily for a period of 14 days.

Results

Virus isolation—Virus induced cytopathic effects (CPE) were characterized by rounding, ballooning of cells, increased refractility and detachment of PLT cells. CPE was initiated by day 2-3 post infection (PI) and progressed to 80% by day 5-6. Virus was confirmed by capripox virus-specific PCR, which amplified a product of 390 bp (Fig. 1).

PCR and Sequence analysis of P32 gene—PCR using the primer pair A95 and B7 yielded a product of ≈1000 bp. Sequence of the gene showed a open reading frame (ORF) of 972 bp (GenBank acc# EU835938). The gene showed 100% sequence identity with other SPPV, followed by 98.5% and 97.7% identity with LSDV and GTPV respectively at nt level. Similarly, the deduced aa sequence identity was >99%, 97.2% and 95.4% with SPPV, LSDV and GTPV, respectively (Table 1).

Animal observations—At 6th passage, virus was inoculated into susceptible sheep for producing the experimental infection. Animal started signs of disease as early as 2 days with moderate rise in rectal temperature. From 4th day onwards, local sites of inoculation showed hyperaemia accompanied with

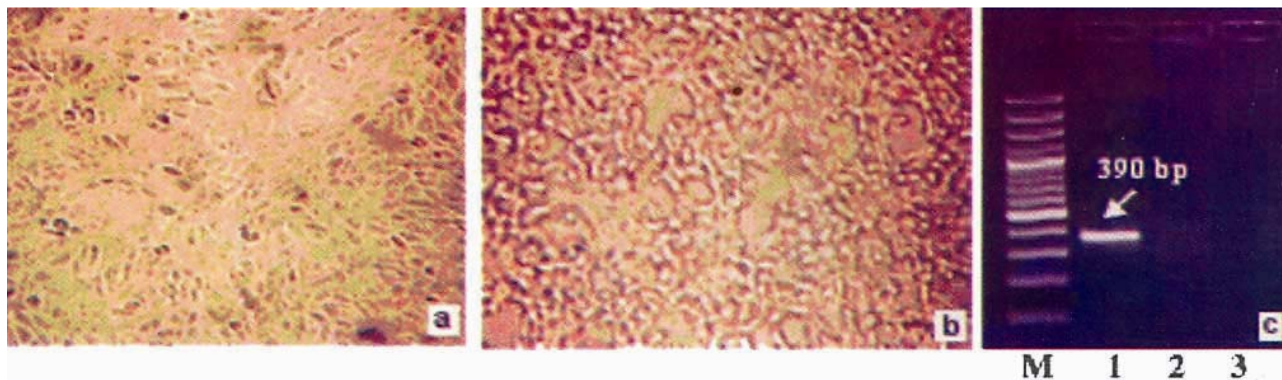

Fig. 1—(a) Cytopathic changes of sheeppox virus (Srinagar) in primary lamb testes. Healthy mock infected secondary cell culture (10x); (b) SPPV (Srinagar) infected cells showing characteristic rounding, ballooning and syncytia with detachment on day 4 post-infection (10x); (c) PCR products from SPPV sample; Lane M: 100 bp DNA molecular size marker, Lane 1: SPPV (Srinagar 38/00) isolate, Lane 2 & 3, Vero cell control.

high rise in rectal temperature (106°C). The severity of the local lesions increased gradually over the next 3-4 days and generalized subcutaneous redness was noticed on day 6-post infection. Animal was pyrexic (106.8°C) (Fig. 2) and off fed from day 5 onwards. Nodular lesions were found on the face, eyelids and groin, though generalized hyperaemia was more prominent than the nodular type of dermal lesions (Figs 3a and 3b). Animal was treated with broad-spectrum antibiotics and analgesics to reduce the clinical severity. Scab samples collected were confirmed by PCR amplification.

Table 1—Percent sequence identity of P32 gene of SPPV-Srinagar 38/00 isolate with other capripoxviruses at the nucleotide (nt) and amino acid (aa) levels.

Sl. No	Virus strain	nt level	aa level
1	GPPV-MU (AY159333)	97.7	95.4
2	GPPV-G20 (AY077836)	97.9	96.3
3	SPPV-RF (AY368684)	99.8	99.1
4	SPPV-Niskhi (AY077834)	100	99.7
5	LSDV-58 (AF325528)	98.2	97.2
6	LSDV-99 (AF409137)	98.5	97.2

Figures in parentheses are GenBank accession number

Validation of the virus for challenge studies—When two batches of vaccines were tested, the vaccinated animals remained normal without showing any disease specific clinical signs, whereas, in contact healthy sheep succumbed to the disease showing rise in body temperature and dermal lesions (Figs 3c and 3d) with similar clinical course as described.

Discussion

In the present study, isolation and characterization of a field isolate of SPPV has been carried out in order to employ a cell culture adapted virulent field sheeppox virus as challenge virus. The virus was associated with a severe outbreak of sheeppox in Srinagar district of

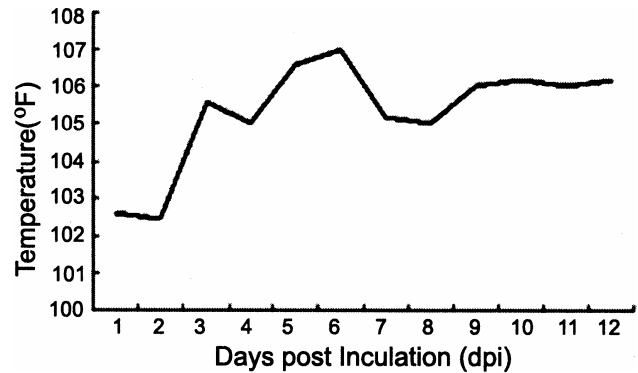

Fig. 2—Mean rectal temperature of the sheep following experimental infection with SPPV

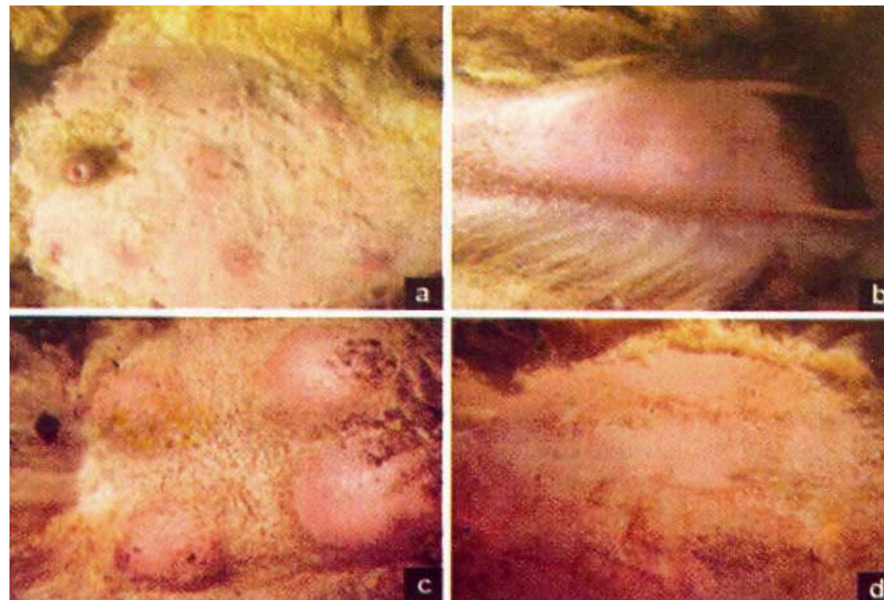

Fig. 3—(a) Sheep showing dermal lesions on the lower abdomen at the site of inoculation on day 4 post infection; (b) and on the abaxial surface of the tail with profuse erythematous lesions in subcutaneous tissue on day 5 post infection on experimental infection with SPPV-SRIN 38/00 (passage 6); (c) In contact sheep showing dermal lesions on the lower abdomen at the site of inoculation on day 4 post infection; (d) No takes were observed in sheep vaccinated and challenged on 28th day post vaccination .

Jammu and Kashmir during August 2000. Virus isolation was carried out in lamb testes cell culture and the virus produced visible CPE in the second cell culture passage. Virus harvested at 80-90% cytopathic changes was further confirmed by diagnostic capripox-specific PCR. Virus was passaged till 6 passages and subsequently used to produce experimental infection in sheep.

Nucleotide sequence of the P32 gene amplicon showed an ORF of 972 bp like in other SPPV, which is 969 bp in GTPV. Members of capripoxviruses are highly related among themselves antigenically while phylogenetically they are distinct[12]. Molecular methods such as duplex PCR[13] and identification of signature residues in the sequence of immunogenic gene are often used for differentiation[14]. Many signature residues that are unique to SPPV including L62F, S132L and I134T were identified in the deduced amino acid sequence of the protein. The virus showed high sequence identity with SPPV having 99.8% and >99.1% identity at the nt and aa level, respectively.

The cell culture adapted virus at passage 6 was used to produce experimental infection in sheep by dermal inoculation. Initially, animal remained apparently healthy for the first three days, despite moderate rise in rectal temperature as early as day 2 PI and no local reactions were evident at the site of inoculation. Generalized hyperaemia was observed from 4-5 day of infection, as evident from subcutaneous redness, although external raised dermal lesions were moderately observed. Animal showed only little improvement in spite of rehabilitation, initiated on day 7-post infection. Similar results were obtained in in-contact animals when two batches of sheeppox vaccines were tested. The vaccinated animal remained apparently normal. Broad-spectrum antibiotic and analgesics were administered to reduce the animal suffering.

Though protection induced upon sheep pox vaccination can be confirmed either by assessing cell mediated and/or humoral immune responses, challenge study is the conclusive to ascertain the protection[15,16]. Therefore, it is required to maintain virulent challenge virus in the laboratory for vaccine efficacy testing. The present study showed that the cell culture adapted virus (6th passage) retained its virulence showing characteristic capripox disease on experimental infection in sheep and may be used in potency testing of sheep pox vaccine. Its application should overcome the disadvantages of using of homologous animals for propagation and titration of virulent challenge sheeppox virus. The virus thus characterized has the potential application as challenge virus for the potency testing of various live attenuated sheeppox vaccines currently used in India.

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