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Isolation and adaptational studies of native Sheeppox Virus strains in lamb testicular and Vero cell culture systems

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Abstract

Sheeppox is a highly contagious disease of sheep causing huge economic losses to the sheep industry and farmers rearing the livestock. Himachal Pradesh has large population of sheep reared by the different rural communities. In recent years, outbreaks of sheep pox have become frequent causing huge economical losses to sheep breeders. Isolation, identification and characterization of circulating Sheeppox virus (SPV) strains is required to initiate efforts for disease control and prevention. The aim of the present study was to establish the best cell culture-based isolation system by comparing the growth of SPV field strains from Himachal Pradesh in lamb testicular (LT) cells and continuous Vero cell line. The results showed that LT cell culture is more suited for primary isolation of SPV field strains as compared to Vero cells. But virus can be adapted to Vero cell line after 5 blind passages. Thus, it was concluded that LT cell culture, though tedious, should be preferred for primary isolation of SPV field strains.

Key words: Sheeppox virus, LT cell culture, Vero cell line.

Sheeppox is one of the major economically important disease of sheep caused by Sheeppox virus (SPV), a double stranded DNA virus of *Capripoxvirus* genus, subfamily *Chordopoxvirinae* and *Poxviridae* family (King *et al*. 2012). The *Capripoxvirus* genus has other closely related viruses namely Goatpox virus and Lumpy Skin Disease virus infecting goat and cattle, respectively. The disease is endemic in the Middle east, including Iran, Afghanistan, Egypt, North Africa, Turkey, Iraq and Indian subcontinent (Bhanuprakash *et al*. 2006). The virus spreads *via* direct contact with diseased animals or by aerosol route. Young lambs of 4 to 5 months age are more severely affected than adults (Malik *et al*. 1997). Infection leads to pyrexia, nasal discharge, ocular discharge, pock lesions throughout skin and mucous membrane (Mirzaie *et al*. 2015). The disease is characterized by fever, loss of appetite, eruption of pox lesions on wool less area of skin such as lips, cheeks, nostrils, abdomen, inner aspect of thigh, and under the tail region.

The virus can be identified by isolation, serological and molecular methods. Isolation of SPV can be done using different live cell systems of various mammalian species. SPV is host specific. Sheep is the natural and highly specific host for the propagation of virus but use of embryonated eggs was also frequent for isolation, characterization and attenuation of SPV in earlier days by many workers. Isolation of SPV was also attempted in chicken embryo fibroblast (CEF) (Ramyar and Hessami, 1967). The Ranipet strain was found to be adapted to CEF (Jassim FA, 1982). With recent advances, embryonated eggs were replaced by cell culture methods as the laws regarding use of animals have become more stringent. SPV was propagated in testicular tissue fragments in earliest attempts and then skin was used for propagation of vaccine strains and cultivated the virus for up to 15 passage as safe and immunogenic (Agyun, 1955). Boue *et a*l. 1957 reported the multiplication of SPV in

ovine kidney and skin cells. Great progress has been made in the propagation of different viruses using tissue-culture methods since Plowright and Ferris (1958) and Cilli and Baldelli (1959) successfully adapted the virus to different cell cultures and studied its characteristics *in-vitro*.

In recent years, outbreaks of sheeppox have become frequent among migratory flocks and native breeds causing huge economical losses to sheep breeders of Himachal Pradesh as well as surrounding states (Batta *et al*. 1999; Verma *et al*. 2011; Sharma *et al*. 2013). Isolation, identification and characterization of circulating field strains SPV is required to initiate efforts for disease control and prevention in Himachal Pradesh. The aim of the present study was to establish the best cell culture-based isolation system by comparing the growth of SPV field strains from Himachal Pradesh in lamb testicular (LT) cell culture and cancerous continuous Vero cell line.

Materials and Methods

Virus

The virus was originally collected in the form of diseased clinical samples from 3 outbreaks of sheeppox in different parts of Himachal Pradesh, where adult sheep was infected. Clinical samples including scabs, nasal swabs, skin tissues were placed in Phosphate buffered glycerine (PBG) for isolation of virus.

Cell lines:

a. Lamb testicular (LT) cell culture: The cells were prepared in laboratory and propagated in Dulbecco modified essential medium (DMEM) containing 10mM HEPES, 2mM glutamine (HiMedia, India) and sodium bicarbonate supplemented with 10% Foetal bovine serum (FBS) (HiMedia, India). Antibiotic cocktail containing mixture of 10,000U/ml penicillin, 10 mg/ml streptomycin and 25µg/ml amphotericin B (HiMedia, India) was also added to avoid microbial contaminations.

Preparation of LT cell culture:

Primary lamb testicular cell culture were prepared using testicular tissues of 3 months old lamb. Testes were obtained aseptically from 3 months old lambs and maintained during transportation in Phosphate buffer saline (PBS) containing antibiotic antimycotic solution (10,000U penicillin/ml, 10mg/ml streptomycin and 25µg/ml amphotericin B (HiMedia, India). Testicle was dissected to remove the fat layer and superficial layers, which was further processed by mincing and incubation with 100 ml of 0.25% trypsin (HiMedia, India) until the cortex was completely digested. The supernatant from repeated digestions was filtered through sieves and cells were collected by low-speed centrifugation (1500 rpm for 10 min). Lamb testicular cells were seeded into 150 cm^2 cell culture flask sat 3×10^6 cells/ml (Corning, USA) and maintained in Dulbecco Minimum Essential Medium supplemented with 10% FBS and antibiotic antimycotic solution containing 10,000U penicillin/ml, 10 mg/ml streptomycin and 25µg/ml amphotericin B for up to 7 days, afterwards the cells were frozen in liquid nitrogen for subsequent use.

b. Vero cell lines: Vero cells are derived from the kidney of an African green monkey and is one of the commonly used mammalian continuous cell line for viral isolation. Vero cell line was procured from the National Centre for Veterinary Type Culture (NCVTC), Hisar. The cells were propagated in Dulbecco Minimum Essential Medium (DMEM) containing 10mM HEPES, 2mM glutamine and sodium bicarbonate supplemented with 10% Foetal bovine serum (FBS).

Preparation of viral inoculum

Tissue collected in PBG were minced in Dulbecco Minimum Essential Medium (DMEM) containing antibiotics, penicillin and streptomycin (HiMedia, India) using sterile sand and pestle and mortar followed by centrifugation at 5000 rpm for 10 min. and filtration of the supernatant through 0.45 µm nitrocellulose membrane filter. Presence of bacterial contamination was checked by overnight culture of the inoculum on the blood agar medium (HiMedia).

Infection of cell lines

LT cells and Vero cells were seeded on 25 cm^2 cell culture flasks (Corning, U.S.) in duplicates using DMEM, FBS (10%) and incubated in incubator at 37°C with 5% CO_2 till the formation of 80 to 90% confluent monolayer. Then after washing the monolayer twice with PBS, 200µl viral inoculum was added and flasks were gently rocked for 60 mins to adsorb virus on the surface of LT/Vero cells. Sterilized 200µl PBS was added to make the uninfected controls. After one-hour rocking, inoculum was removed and

5ml cell medium DMEM, FBS (10%) along with antibiotics was added. Cells were daily examined for appearance of cytopathic effect (CPE) for 7 to 14 days after infection. Subsequent 3 to 5 passages were given in each cell culture till the CPE were observed. The virus isolates were preserved at -80°C for further experimentation.

Confirmation of virus growth by *P32* gene-based PCR

DNA was extracted from the LT and Vero cells grown virus from cell suspensions harvested after the appearance of CPE using Qiagen DNA extraction kit (Qiagen, Germany) as per manufacturer protocol. The extracted DNA samples were tested with PCR using conditions as reported earlier i.e., *P32* gene based *Capripoxvirus* group specific PCR with B68 and B69 primers (Heine *et al*. 1999).

P32	B68	CTAAAATTAGAGAGCTATACTTCTT
	B69	CGATTTCCATAAACTAAAGTG

Results and Discussion

Tissue culture systems have become the convenient way of the propagation of viruses and studying the biology and pathogenicity of viruses. Suitable cell culture system is also required for production of antigens for diagnostic development as well as for production of attenuated vaccines since the regulations for use of animals have become stringent. Sheeppox virus can be propagated in different cell types of many host origins with varying susceptivity. Cell lines of ovine, caprine and bovine origin can be used (Pandey and Singh, 1970; Mateva, 1976). In our study, we used lamb testicular cell culture which, we prepared in our laboratory using testicular tissues of a young lamb. The lamb testicular cell is most widely used system for the preparation of vaccine strains.

In our experiment, we selected host specific LT cells of sheep origin and Vero cells of monkey origin. We infected the cells with virus in clinical samples (n=12) and observed the cells for cytopathic effects. On first passage, visible cytopathic effects (CPE) were produced by the virus on 13th day of incubation (Fig 1A). In 3rd passage, characteristic CPE appeared as early as 72 hrs along with the lysis of LT cells. Rounded, refractile cells in isolated foci were also found. Cells free spaces were found in monolayer due to cytoplasmic retractions. Passage number and incubation days wise appearance of CPE details are shown in Table 1. After subsequent passages, there was increased refractivity of cells (Fig 1B) and early lysis of cells were observed. The control cells remained unchanged in cytomorphology (Fig 1C). SPV showed no growth in Vero cells till passage 4. CPE started its appearance in Vero cells on 13th day of incubation in passage 5th. Out of 12 samples, all the samples were successfully processed to grown SPV on LT cells but only 4 viruses were able to multiply in Vero cells.

In the second set of experiment, virus isolated on LT cells was inoculated on Vero cells and 5 blind passages were given. The virus adopted to the Vero cells after 5 passages. The sequence of CPE appearance is shown in Table 2. There was no change in initial passage of virus in Vero cells (Fig 2A). CPE were observed after 5th passage of virus in Vero cells (Fig 2B). There was formation of round syncytia of cells. Morphology of control cells remained unchanged (Fig 2C).

Confirmation by PCR

Successful isolation of the SPV in LT and Vero cells at the stage of CPE appearance was confirmed by virus specific *P32* gene-based PCR screening. The SPV specific amplicon of 389 bp was detected in all samples those shown CPE at particular stage of incubation (Fig 3).

Sheeppox is a highly contagious disease of sheep causing huge economic losses. The detection of virus is of prime importance for control of disease in that case isolation of virus becomes important for further characterization. Earlier, embryonated chicken eggs and target specie (sheep) were used for the propagation of virus. These methods are discontinued now owing to the availability of more convenient and humane methods like tissue and organ culture. Susceptibility of various cell types for different viruses and their field strains varies (Panday and Singh, 1970). Choice of suitable tissue culture system is essential for convenient propagation of viruses for studying their biology, pathogenesis, molecular epidemiology and genetic variations. Further, production of attenuated viral vaccines also requires suitable cell adopted viruses.

For propagation of SPV, tissue culture of ovine origin is frequently used. Commonly used SPV

Table 1. Details of CPE appearance in LT and Vero cells at different passage levels during the incubation upto 14 days

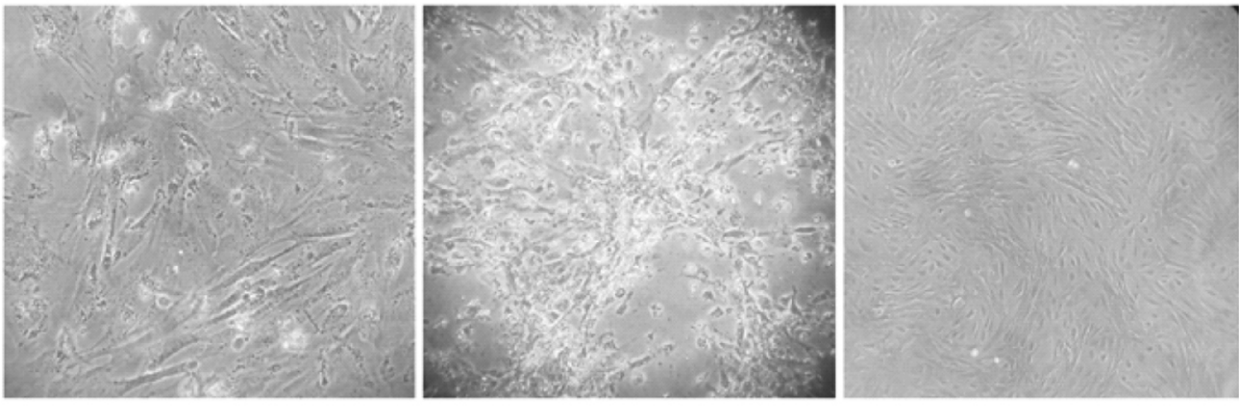
Cell Type	Days of Incubation														Passage Interval
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
LT	-	-	-	-	-	-	-	-	-	-	-	-	±	+	P1
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LT	-	-	-	-	-	-	±	±	±	+	+	+	+++	+++	P2
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LT	-	-	+	+	+	+	++	+++	+++	+++	+++	+++	+++	+++	P3
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LT	-	-	+	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	P4
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
LT	-	-	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	P5
Vero	-	-	-	-	-	-	-	-	-	-	-	-	±	±	
LT	-	-	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	P6
Vero	-	-	-	-	-	-	-	-	-	-	-	-	±	±	

Note: (-) No CPE; (±) appearance of CPE; (+) scattered CPE; (++) moderate CPE; (+++) represents cell lysis

Table 2. Details of CPE appearance in Vero cells at different passage levels after the inoculation of SPV isolated initially on LT cells

Cell Type	Days of Incubation														Passage Interval
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P1
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P2
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P3
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P4
Vero	-	-	-	-	-	-	-	-	-	-	-	-	-	-	P5
Vero	-	-	-	-	-	±	±	±	+	+	+	+	++	++	P6

Note: (-) No CPE; (±) appearance of CPE; (+) scattered CPE; (++) moderate CPE; (+++) represents cell lysis



A B C

Fig 1. Virus growth in LT cells. A: LT cells in 3rd passage; B: LT cells in 6 passage; C: LT Control cells after 7 days of incubation

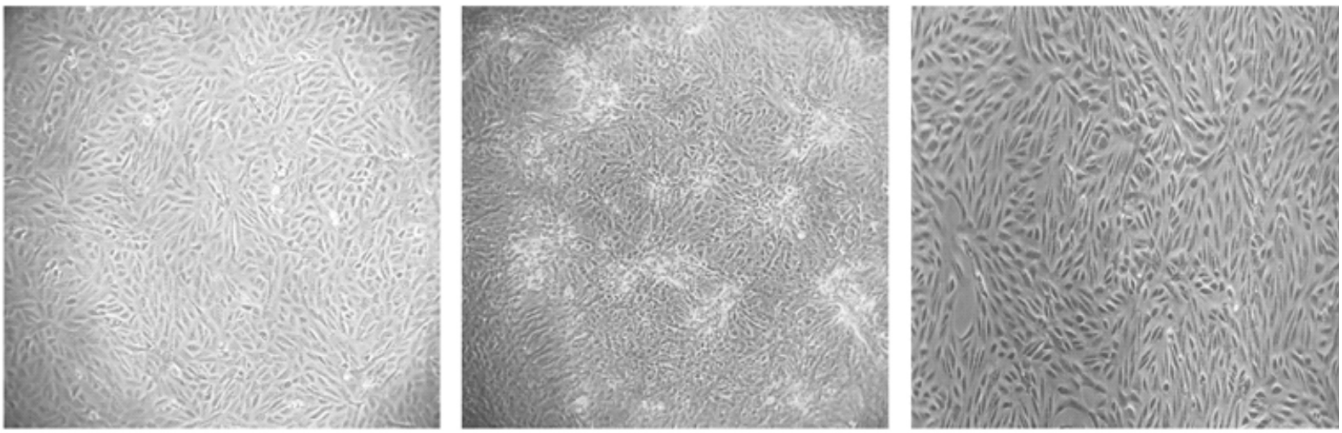


Fig 2. Virus growth in Vero cells. A: Vero cell in I[st] passage; B: Vero cell in 5 passage; C: Vero cell control after 7 days of incubation

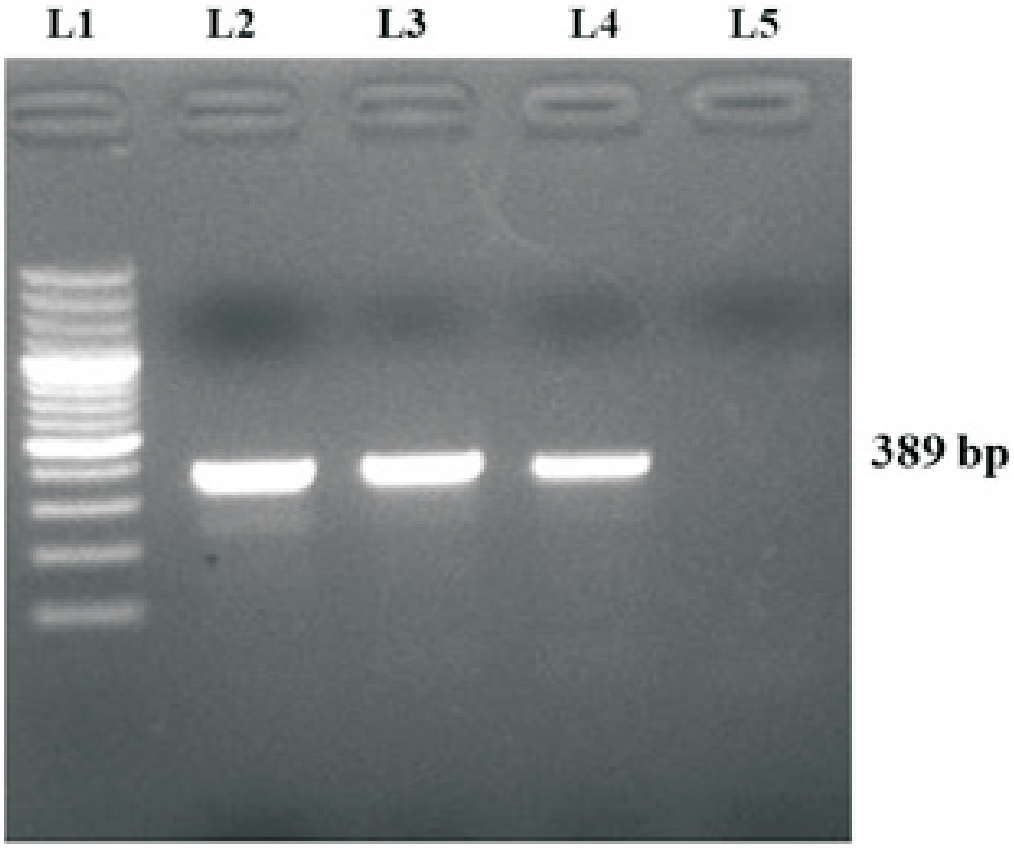


Fig 3. Confirmation of Sheeppox virus by *P32* gene-based PCR. L1: 100 bp ladder; L2: LT cells grown virus after 3 passages; L3: LT adapted SPV grown in Vero cells; L4: Positive control of SPV; L5: negative control

vaccine strains are attenuated in primary or secondary lamb testicular cell line. For studying the suitability of LT and Vero cells to isolate and propagate native SPV strains from Himachal Pradesh, clinical samples collected from field outbreaks of Sheeppox were used. The appearance of cytopathic effects and confirmation of viral isolation results varied in different studies. Soman and Singh (1980) observed first CPE 72hrs of infection in low passage in lamb kidney and lamb testicular cell lines, where it was higher in percent in lamb testicular cells. The cytopathic changes depend on high or low titre of virus in inoculum (Coakley and Capstick, 1961). Many workers have used continuous cell lines for the cultivation of virus. Primary or secondary ovine cell line requires require fresh stock of cells isolated from animal, which is labour intensive, expensive and in-humane, so continuous cell line system becomes essential routine in cultivation of SPV. Baibuk (2007) found the continuous ovine testicular cell line as more effective than primary lamb testicular cell line. Some scientists were successful in adapting SPV in BHK-21 and Vero cells (Kirubaharan *et al*. 1993). Virus was able to replicate properly in Vero cells after 9 passages, when LT adapted virus was grown in that cell line (Trabelsi *et al*. 2014) but in our study, native SPV strains adopted to grow in Vero cells after 5 passages. Vero cells have been used for the preparation of sheeppox vaccine using Srinagar strain. Goatpox virus was also propagated in Vero cells for the production of vaccine (Hosamani *et al*. 2004). Singh and Rai adapted the Jaipur strain to Vero cell cultures, where CPE initiation was observed at 24 hrs, which reached a maximum after 96 to 120 hrs on the 5th day.

The appearance and adaptation of virus to cell system varies on many factors such as initial concentration of virus and strain of virus. In our study also, clinical samples usually with low virus load yielded no observable CPE after 5 passages in Vero cells but CPE appeared earlier in LT cells. CPE started appearing on 5th day after inoculating higher titre of LT adopted SPV on 6th passage in Vero cells. In our study, native the strain is easier to isolate on LT cells even in low viral load as compared to Vero cell.

Conclusion

Primary cell line of lamb testicular origin was better for both the growth and maintenance of the SPV. However, Vero cell line could also sustain the growth of virus only when it was inoculated in higher titres and was already adapted on LT cells. It is advisable to use continuous cell lines from other hosts like Vero cell line (monkey origin) for cost effective and convenient propagation in laboratories for further studies.

Conflicts of interest: The authors declare that there is no conflict of interest in this research paper.

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