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# Isolation of Lumpy Skin Disease Virus from Andhra Pradesh

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**ABSTRACT**

A total of 31 blood samples and 31 skin scabs were collected from both adult and young calves of suspected cases of Lumpy skin disease showing clinical signs. All the collected samples (31 blood samples and 31 skin scabs) were processed and subjected for virus isolation in embryonated chicken eggs and primary testicular cell cultures. The skin scabs (7 out of 14) positive at genus and (amplifying P32 gene) species level amplifying (GPCR, RPO30 and Ankyrin repeat) genes by PCR were attempted for virus isolation on embryonated chicken eggs of 10-11 days old. All the seven virus isolates (TPT-1, TPT-2, K-1, K-2, G-1, CH-1, CH-2) showed hemorrhages on chorio-allantoic membrane at second passage level on 5$^{th}$ day of post inoculation whereas only three isolates (TPT-2, K-1, CH-1) showed characteristic pock lesions on 5$^{th}$ day of post inoculation at 5$^{th}$ passage level. These were further confirmed by PCR amplifying P32 gene. Out of these three isolates (TPT-2, K-1, CH-1) only two isolates (TPT-2, K-1) were subjected for adaptation in primary lamb testicular cell cultures and CPE was observed with shrinkage, cell rounding and cell aggregation between 48-72hrs post infection at first blind passage level.

**HIGHLIGHTS**

- Isolation of Lumpy skin disease virus from Blood and Skin scabs.
- Isolation of Lumpy skin disease virus in Embryonated chicken eggs and primary testicular cell cultures.

**Keywords:** Lumpy skin disease virus, Embryonated chicken eggs, primary testicular cell culture

LSD is an important notifiable disease and hampers the international trade (EFSA, 2015; Abutarbush *et al*., 2013; Babiuk *et al*., 2008a). Lumpy skin disease virus is recently considered as a potential agent of agro terrorism because of its endowed ability to spread. During an outbreak in Jordan the estimated average cost of supportive antibiotic treatment to be 27.9 British pounds per head (Abutarbush *et al*., 2013). The annual financial cost included the average production losses, due to morbidity and mortality arising from milk loss, beef loss, traction power loss, treatment and vaccination costs at the herd level. The average financial cost in infected herds was estimated to be 6.43 USD per head for local zebu cattle and 58 USD per head for Holstein Friesian or crossbred cattle (Gari *et al*., 2011).

LSD is a non-zoonotic, vector borne and trans boundary disease with limited host range and currently restricted to ruminants viz. cattle and water buffaloes. However caprine and ovine species have been able to resist infection even when together in close proximity to infected buffaloes and cattle. Nevertheless, certain experimental cases have been observed in laboratory settings whereby skin lesions were produced in a wide assortment of wild and domestic animals i.e. giraffes, impalas, gazelles, sheep, and goats (Davies, 1991; Padilla *et al.,* 2005).

Diagnosis is by virus isolation and molecular methods which will not only confirm the presence of virus and

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also revealed the origin of virus and its relationship with other virus isolates worldwide. Virus isolation can be used for the confirmatory diagnosis in new niches. The bovine testes and pre-pubertal lamb, primary and secondary cultures are most sensitive systems for isolation of virus (OIE, 2017).

## MATERIALS AND METHODS

### Collection of samples

The clinical samples were collected from suspected cases of LSD during the outbreaks in different districts of Andhra Pradesh from April 2021 to November 2021. Samples were collected from both adult cattle and young calves based on typical signs of LSD shown by the animals. Blood samples were stored at -4 °C or at -20 °C, whereas the tissue samples were stored at -70 °C until further processing.

### Blood Samples

A total of thirty one (31) blood samples were collected from clinically suspected cases of LSD at the pyrexia stage. Detailed collection of history and physical examination was done on suspected animals before the collection of samples (Table 1).

### Skin Scabs

A total of 31 skin scabs were collected in 10% glycerol saline during outbreaks in Andhra Pradesh from skin lesions of suspected LSD cases. The list of samples collected were mentioned in the Table 1.

## PROCESSING OF SAMPLES FOR VIRUS ISOLATION

### Processing of blood samples

Initially the blood samples collected from suspected cases was processed to separate the buffy coat from the unclotted blood using centrifugation at 600 g for 15 min after addition of lymphoprep buffer and the buffy coat separated was carefully added into 5 ml of cold double distilled water using a sterile pasteur pipette. After 30 seconds, 5ml of cold double strength growth medium was added and mixed thoroughly. Then the mixture was centrifuged at 600 g for 15 minutes, the supernatant was discarded and the cell pellet was suspended in 5 ml of growth medium (MEM). The mixture was again centrifuged at 600 g for 15 minutes and the supernatant was discarded and the resulting pellet was suspended in 5 ml of fresh MEM and stored at -70 °C until further use. (OIE, 2017).

### Processing of skin samples

Skin scabs for virus isolation was done in horizontal laminar air flow. The skin scabs collected from affected clinical cases were cut into small pieces using sterile scalpel blade and forceps and grounded with a pestle in a sterile mortar with sterile sand after addition of an equal volume of sterile phosphate buffered saline (PBS) or serum-free modified Eagle's medium as 10 fold dilution

**Table 1:** Details of samples collected for isolation of LSDV from Andhra Pradesh

| Sl. No. | Place of collection | Kind of species | Kind of samples collected | No. of samples collected |
|---|---|---|---|---|
| 1 | TVCC, Tirupati, Chittoor district | Bovine | Blood | 4 |
| | | | Skin scabs | 4 |
| 2 | Mahanandhi, Kurnool district | Bovine | Blood | 10 |
| | | | Skin scabs | 10 |
| 3 | Livestock Research Station, Lam Farm, Guntur district | Bovine | Blood | 5 |
| | | | Skin scabs | 5 |
| 4 | Puttur district | Bovine | Blood | 8 |
| | | | Skin scabs | 8 |
| 5 | Pallavaram, Tirupati Rural | Bovine | Blood | 4 |
| | | | Skin scabs | 4 |
| **Total** | | **(Blood-31), (Skins cabs-31)** | | **62** |

(10%) along with sodium penicillin (1000 international units [IU]/ml), streptomycin sulphate (1 mg/ml). The suspension is freeze– thawed three times and then partially clarified by centrifugation using a bench centrifuge at 600 g for 10 minutes. The supernatant was filtered through a 0.45 µm pore size filter after the centrifugation step and the viral suspension was stored at -70 °C until further use (OIE, 2017).

## VIRUS ISOLATION

### Isolation in embryonated chicken eggs

Specific pathogen free embryonated chicken eggs of 10-11 days old were procured from Balaji Hatcheries farm, Chittoor, Andhra Pradesh and used for virus isolation. 0.2 ml of inoculum from 10% tissue suspensions of infected skin scabs was inoculated into 10-11 day old chicken embryos through chorio-allantoic membrane (CAM) route followed by incubation at 37°C in an egg incubator for 5 days and the eggs were observed daily for changes. The eggs died within 24 hrs were treated as non-specific death and removed. After incubation of 5 days, eggs were chilled at 4°C for 4-6 hrs for the death of embryos and to avoid bleeding. After death of the embryo, the infected CAM, were collected aseptically. Further, the CAMs were triturated in pestle and mortar with sterile sand and made 10% suspension in PBS containing antibiotics, and centrifuged at 3000 rpm for 10 min. The supernatant was collected and filtered through 0.45 µm filter and was stored at -20°C for further use. Later next 0.2 ml of infected CAM suspension thus prepared was used for next batch of embryos for serial passage. Similar five passages were carried out to isolate the virus in embryonated chicken eggs and finally CAMs were collected from the embryos with characteristic pock lesions.

### Adaptation of LSDV field isolates to primary lamb testicular cell cultures

In order to adapt LSDV field virus isolates to primary lamb testicular cell cultures, passages of embryonated chicken eggs about 0.2 ml of virus suspension was inoculated into the monolayer and incubated for 2 hours at 37 ºC for adsorption. Following adsorption, washed with maintenance medium to remove the unadsorbed virus. Then added with maintenance medium and incubated at 37 ºC in 5% $CO_2$ tension. The infected cell cultures were examined daily cytopathic effect. Later the monolayers showing 75% CPE, were taken and stored at -70 ºC to identify the presence of virus.

Molecular confirmation of LSDV field isolates recovered from embryonated eggs and primary lamb testicular cell cultures

All the recovered LSDV field isolates on embryonated chicken eggs and primary monolayer cell cultures were subjected to conventional PCR for the identification of LSDV amplifying genus specific primers targeting P32 gene.

## RESULTS AND DISCUSSION

### Clinical findings

The most common clinical signs of LSD observed in affected cattle during clinical examination includes fever (above 40 ºC) which persisted for 3-4 days, salivation, ocular and nasal discharges followed by edema and enlargement of the superficial lymph nodes, a marked reduction in milk production and skin lesions (1-5 cm in diameter), mainly on the head, neck, legs, udder, tail and back immediately after the febrile stage. In affected animals the nodules were distributed all over the body (Fig. 2). These skin nodules were ruptured and became deep seated wounds with the involvement of all the layers of the skin which paves the way for secondary bacterial infections which led to extensive suppuration and sloughing resulted in hard, raised areas (sit-fasts) clearly separated from the surrounding skin (Fig. 1). Most of the animals recovered except few which died due to extensive lesions, anorexia and emaciation especially young calves.

**Fig. 1:** LSD with deep seated wounds

**Fig. 2:** Characteristic of LSD with generalized circumscribed skin nodulescovering the entire body

During the study period where LSD cases were reported, a total of thirty one (31) blood samples and thirty one (31) skin scabs were collected from cattle with typical signs of LSD from different districts of Andhra Pradesh and subjected for virus detection as well as attempted virus isolation.

Virus isolation was carried out with conventional isolation methods such as embryonated chicken eggs and primary lamb testicular cell cultures. Out of fourteen skin scabs which were positive on PCR to capripox.

Virus primers and GPCR, RPO30 and Ankyrin repeat genes, only seven samples (skin scabs) TPT-1, TPT-2, K-1, K-2, G-1, CH-1, CH-2 were attempted for virus isolation on fertile specific pathogen free embryonated chicken eggs. The 10% tissue suspension was made from these skin scabs with addition of antibiotics, filtered through 0.45 µm filters and 0.2 ml was inoculated into the chorio-allantoic membrane of 10-11 days old embryonated chicken eggs. All the infected eggs showed hemorrhages on the chorio-allantoic membranes on 5$^{th}$ day at 2$^{nd}$ passage level. But out of seven infected eggs three (TPT-2, K-1, CH-1) were showed characteristic small opaque white focal pock lesions on the chorio-allantoic membranes on 5$^{th}$ day at 5$^{th}$ passage level. These infected embryos with pock lesions were confirmed further by PCR, amplified P32 gene and yielded 192 bp confirming capripox genus.

After five passages of seven LSD virus isolates in embryonated chicken eggs only three virus isolates shown characteristic pock lesions on CAM, out of these only two isolates (TPT-2, K-1) were attempted for virus isolation and adapted to primary lamb testicular cell cultures for further confirmation of the presence of the virus. Initially these two viral isolates (TPT-2, K-1) doesn't shown any CPE however, CPE was noticed at first blind passage with shrinkage, cell rounding and cell aggregation between 48-72 hrs post infection and complete CPE after 96 hrs of post infection. Similar pattern of CPE was also observed at 2$^{nd}$ passage with same interval of time.

## LUMPY SKIN DISEASE VIRUS ISOLATION

### Isolation in embryonated chicken eggs

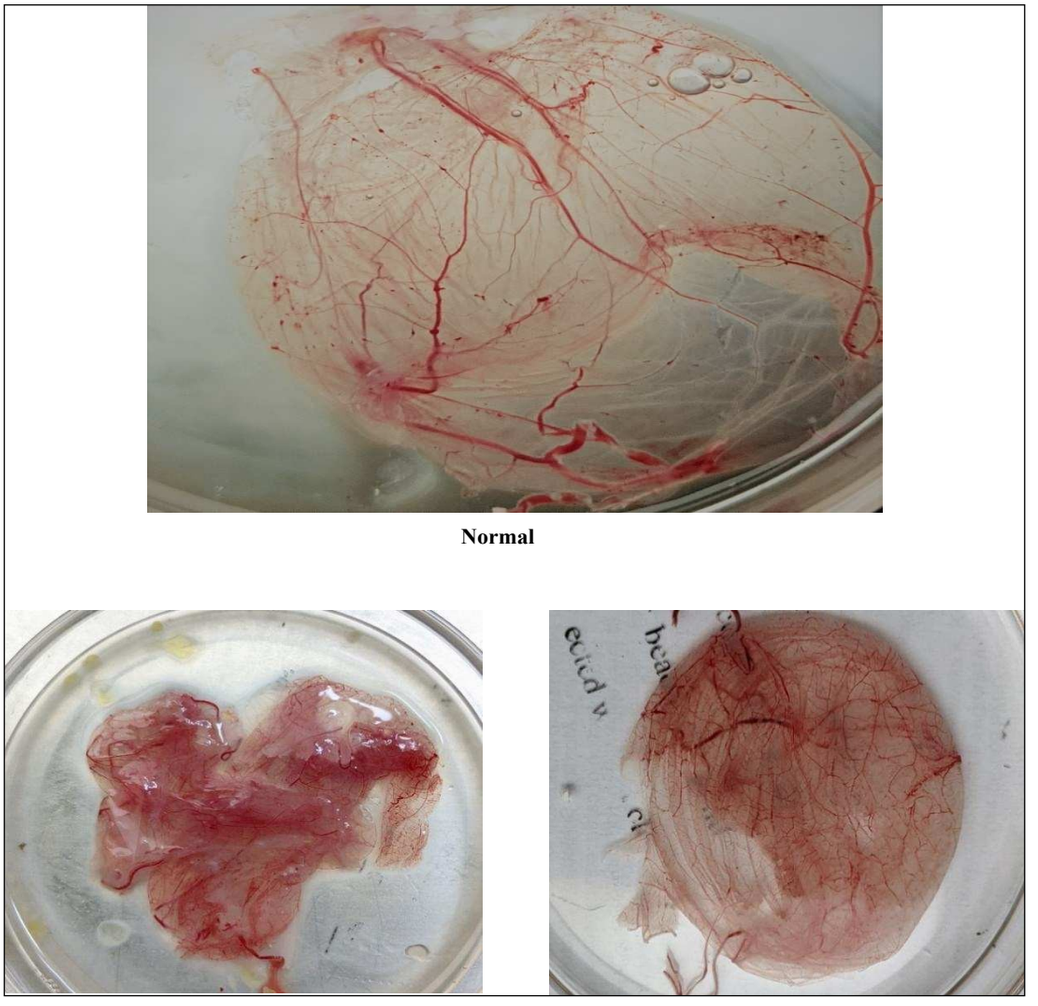
A total of seven positive skin scab samples (TPT-1, TPT-2, K-1, K-2, G-1, CH-1, CH-2) out of fourteen positive skin scab samples which successfully amplified using P32 gene were subjected to infect the specific pathogen free embryonated chicken eggs. The 10% tissue suspension of 0.2 ml inoculum of PCR positive skin scabs was inoculated onto the Chorio-allantoic membrane (CAM) of embryonated chicken eggs (10-11 days) and incubated at 35 ºC under 70% humidity for 5-6 days. The infected embryonated chicken eggs were candled everyday. The dead embryos within 24 hrs would be considered as non-specific and they were discarded. During the period of incubation it was observed that at 2$^{rd}$ passage level, the hemorrhages were observed on CAMs of all the infected eggs (Fig. 3). Whereas, at 3$^{rd}$ passage in one of the infected chicken eggs small opaque white focal pock lesions were observed on CAM. But characteristic small pin point pock lesions were observed in three embryonated chicken eggs (TPT-2, K-1, CH-1) on the 5$^{th}$ day of post inoculation at 5$^{th}$ passage level (Fig. 4; Table 2).

### Confirmation of LSDV recovered from CAMs of embryonated chicken eggs

The infected CAMs showing characteristic pock lesions collected at 5$^{th}$ passage level were subjected to PCR

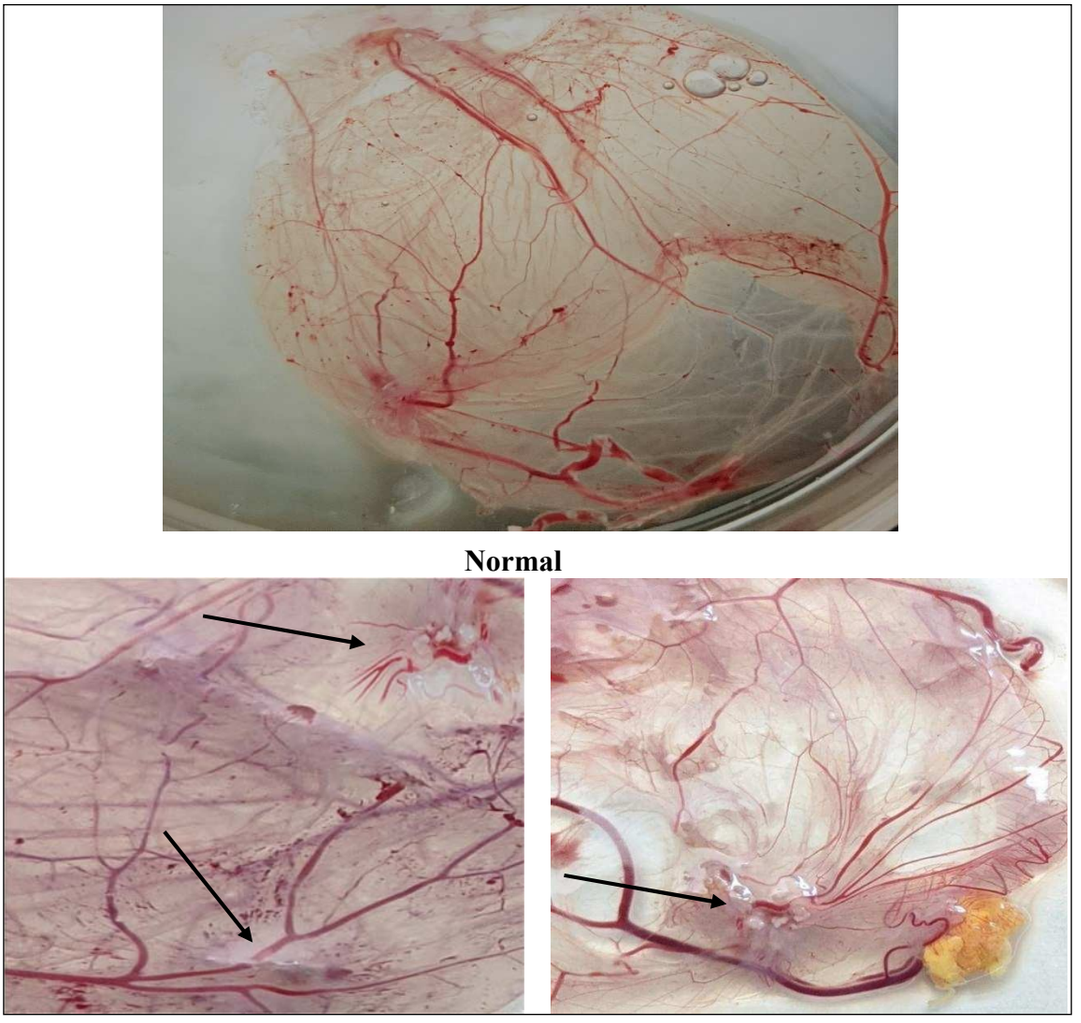
**Table 2:** Details of results of lesions of LSDV isolates on CAM of ECEs

| Sl. No. | Day of observation | Passage level | Gross Lesions on Chorio allantoic membrane |
|---|---|---|---|
| 1 | 5th day | 1st passage | Small pinpoint like hemorrhages are seen at the site of inoculation. |
| 2 | 5th day | 2nd passage | Thickened, congested and hemorrhgic chorio- allantoic membranes were observed. |
| 3 | 5th day | 3rd passage | White opaque focal lesions were seen in one of the infected chorio allantoic membrane |
| 4 | 5th day | 4th passage | Similar white opaque focal lesions along with hemorrhages were observed as in 3rd passage |
| 5 | 5th day | 5th passage | Opaque pin point white focal pock lesions were observed in three of the infected embryonated chicken eggs |




LSDV isolates (TPT-2, K-1) showing hemorrhagic lesions on the chorio-allantoic membrane of embryonated chicken eggs.

**Fig. 3:** Results of LSDV on chorio-allantoic membrane of infected ECEs at second passage




LSDV isolates (TPT-2, K-1) showing characteristic pock lesions on the chorioallantoic membrane of embryonated chicken eggs.

**Fig. 4:** Results of LSDV on chorio-allantoic membrane of infected ECEs at 3rd passage

amplification targeting P32 gene of capripox genus and yielded amplicon of 192 bp in agarose gel electrophoresis indicating that the LSDV was successfully propagated at 5th passage in embryonated chicken eggs (Fig. 5).

### Isolation of LSDV in Primary Lamb Testicular cell cultures

The LSDV infected chorio-allantoic membranes were further adapted to primary cell cultures i.e., lamb testicular cells for virus isolation. Only two virus isolates (TPT-2, K-1) out of seven isolates recovered from infected embryonated chicken eggs were used for virus isolation adapting to primary lamb testicular cells. The confluent monolayer of lamb testicular cells (LT) were infected with virus suspension during first passage did not reveal any CPE for more than 96 hrs of post infection. Upon first blind passage, lamb testicular cells started showing shrinkage, cell rounding, cell aggregation between 48-72 hours post infection and clear CPE was evident at 96-120 hours post infection (Fig. 6).

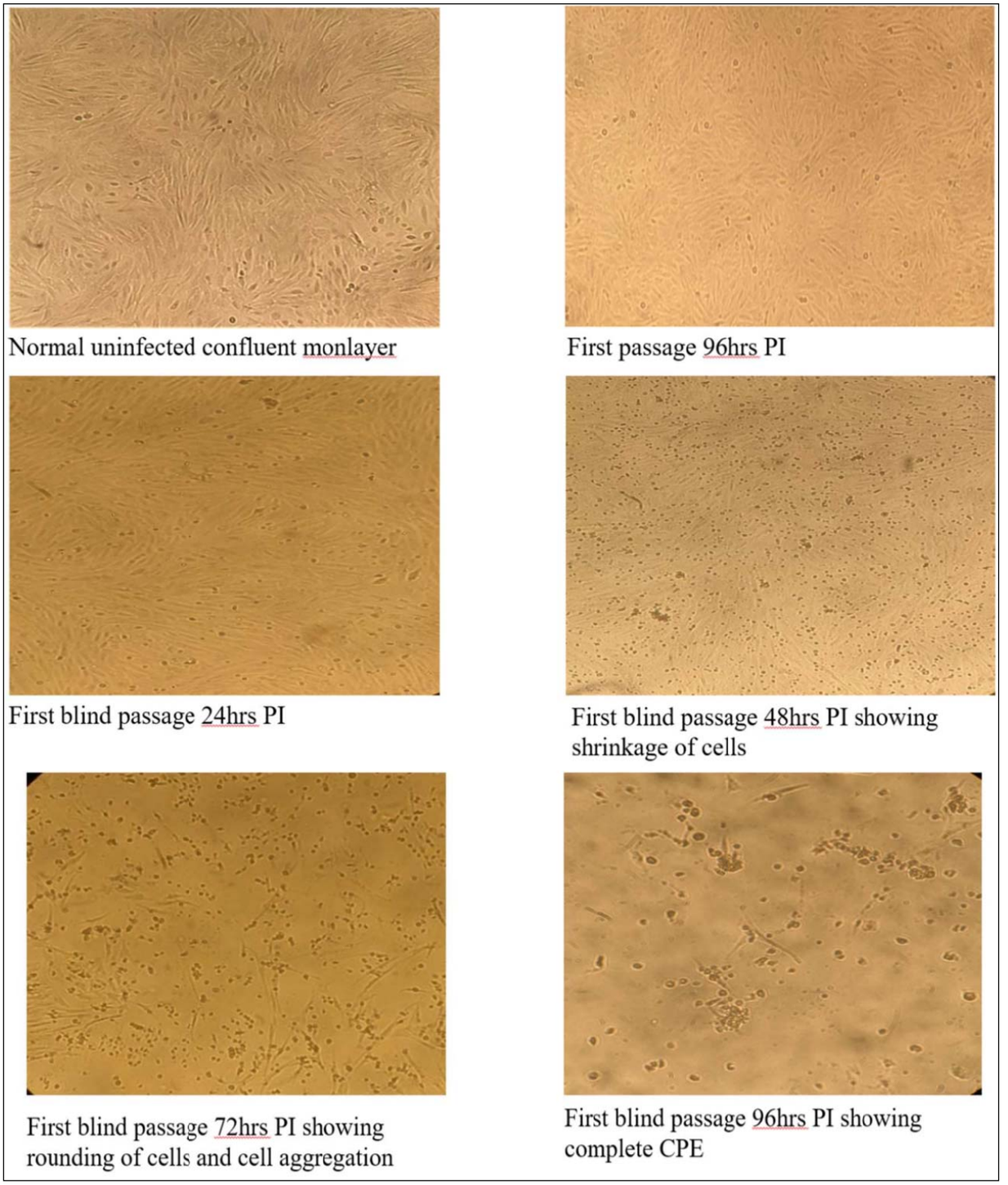
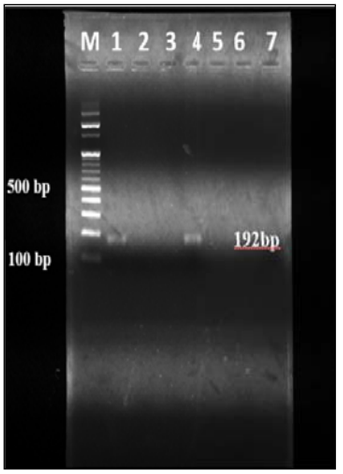
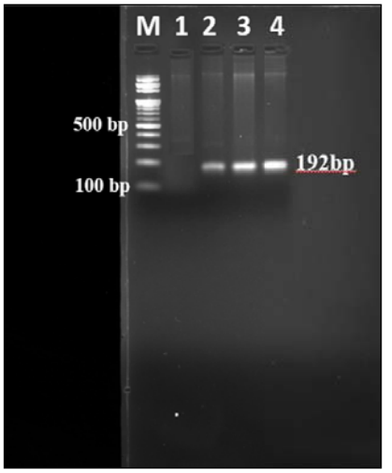



**Fig. 5:** Results of LSDV isolate on primary lamb testicular cell cultures




**Lane M :** Molecular weight marker (100 bp); **Lane 1:** Positive control; **Lane 4 :** LSDV isolate (TPT-2) 192 bp amplicon of *P32 gene;* **Lane 5 :** Negative control

**Fig. 6:** PCR amplification of P32 gene of LSDV recovered from CAM ofembryonated chicken eggs




**Lane M :** Molecular weight marker (100 bp); **Lane 1:** Negative control; **Lane 2 :** Positive control; **Lane 3-4:** LSDV isolates (TPT-2, K-1) 192 bp amplicon of *P32 gene*

**Fig. 7:** PCR confirmation of LSDV recovered from primary lamb testicular cell cultures amplifying P32 gene

**Confirmation of LSDV recovered from primary lamb testicular cell culture lysates**

The replication and presence of virus in the lamb testicular cell cultures were confirmed using the DNA isolated from both infected and uninfected cell culture supernatants with PCR targeting the P32 gene. The positive amplification of 192 bp was observed from the infected cell culture fluid and no amplification was observed from the normal cell culture negative control (Fig. 7). This indicated the successful adaptation and replication of LSDV field isolate in the lamb testicular cell culture.

In the present study, virus isolation was attempted using Embryonated chicken eggs (ECE) via chorio-allantoic membrane route (Tamam, 2006; El Nahas *et al*., 2011; Van Rooyen *et al*., 1969). All the seven samples (TPT-1, TPT-2, K-1, K-2, G-1, CH-1, CH-2) used for isolation in ECE showed hemorrhages at 1st passage level and characteristic pock lesions was observed at 5$^{th}$ passage level (TPT-2, K-1, CH-1) were in agreement with previous reports (Tamam, 2006; El Nahas *et al*., 2011; Aboelkhair *et al*., 2017). However, the absence of lesions is apparently not a reliable indication of virus multiplication according to Van Rooyen *et al.* (1969).

Primary cell cultures of ovine, caprine and bovine origin are usually employed for LSDV isolation. Of these primary bovine dermis and primary lamb testicular cells are considered to be the most susceptible for the cultivation of LSDV (OIE, 2017). Hence, in the present study, primary lamb testicular cell cultures were employed for isolation of the virus and confirmation of the presence of LSDV. After initial isolation of LSDV from embryonated chicken eggs, the LSDV isolates (TPT-2, K-1) were adapted into the primary lamb testicular cell cultures. In the present study the CPE was observed between 48-72 hrs of infection after first blind passage (second passage) itself with characteristic shrinkage, cell rounding and cell aggregation and complete CPE was evinced after 96hrs of post infection. The findings of cell culture propagation and cytopatheic changes caused by LSDV were in similar with observations reported by other workers (Salnikov *et al*., 2018; Orynbayev *et al*., 2020; Arjkumpa *et al*., 2021). During this study CPE was observed in primary lamb testes cell cultures at first blind passage itself when compared to embryonated egg passages which shows lesions at 3$^{rd}$ to 5$^{th}$ passage level. This might be due to the earlier passaging of all field virus isolates primarily in embryonated chicken eggs before adaptation into primary cell cultures. However, a detailed study is required to

analyze the viral replication, cytopatheic changes, and infectivity of the field virus isolates in embryonated chicken and primary cell cultures.

Confirmation of passaged virus from CAM suspensions and cell culture adapted field virus isolates by PCR method using the virus infected cell culture supernatant is the most important one for successful adaptation of field virus in the cell culture system. In the present study, we amplified the P32 gene at first blind passage cell culture supernatant and confirmed the presence of LSDV which were in agreement with the earlier reports (El- Nahas *et al.,* 2011; Salnikov *et al*., 2018; Orynbayev *et al*., 2020).

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