Indian Journal of Animal Sciences **96** (7): 606–608, July 2026/Short communication
https://doi.org/10.56093/ijans.v96i7.172260

Comparative evaluation of culture media for improved yield and viability of primary testicular cell lines from ovine (lamb) tissue

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Received: 01 October 2025; Accepted: 21 July 2026

Keywords: Lamb testicle, Media, Primary culture

Primary cell culture is the *ex vivo* culture of cells freshly obtained from a host, contrary to the culture of immortalized cell lines. In general, primary cell cultures are considered better representatives of *in vivo* tissues than cell lines (Geragthy *et al.* 2014) as they are obtained from host tissue without modification, and they are more similar to the *in vivo* state and exhibit normal physiology (Zhao, 2023). In the field of veterinary diagnosis, the primary cell culture plays a very crucial role in virus isolation and preparation of vaccines. After primary isolation of viruses in a primary cell line, the practice of subsequent adaptation in continuous cell line is a practice in vogue. In recent past, Lumpy skin disease virus (LSDV) belonging to family Poxviridae has caused havoc in the Indian sub-continent leading to catastrophic impact on the livestock population. The virus can be suitably isolated and passaged in array of continuous cell lines, yet the primary cells, more specifically Lamb Testicles, offer an ideal ecosystem for study of viral dynamics, primary isolation, and genotypic studies (Kononova *et al.* 2021). Even other members of the genus such as sheep pox virus (SPPV) and goat pox (GTPV) virus have been usually grown on primary cells for diagnosis, production and titration purposes. The variability induced in primary cells acquired from donors and during subculture practices is a major challenge faced by the researchers who study cell signaling pathways (Lito *et al.* 2020). Researchers prefer to screen the cells for sensitivity to common stimuli before embarking on signaling studies. Pre-screened primary cells save valuable resources as they are stimulated for activation of major signaling pathways. Primary cells require adequate substrate and nutrient conditions to thrive and after a certain number of divisions, they acquire a senescent phenotype, leading to irreversible cell cycle arrest (Campisi *et al.* 2007). However, standardized protocols for efficient isolation and culture of lamb testicular cells are valuable tools in reproductive biology, virology, and livestock biotechnology. Since different culture media compositions significantly influence the isolation efficiency, viability, proliferation rate, and morphological integrity of primary lamb testicular cells, and optimization of media can improve the establishment of stable primary cell lines. The present study aimed to compare different basal media and supplementation strategies for the establishment of viable primary lamb testicular cell lines.

Three months old lamb testicle was collected from local abattoir in aseptic condition, suspended into chilled PBS having 2x antibiotic-antimyotic solution (M/s Himedia) and immediately transported to laboratory at 4°C. The testicle was washed thrice with fresh PBS having 2x antibiotic-antimyotic solution before removal of outer thick layer of fibrous connective tissue (tunica albuginea). The processing and disaggregation of testicle was done as per preparation of primary culture described by Fresheny (2015) with slight modifications. Briefly, the parenchyma of testicle was chopped into fine pieces of tissue using sterile scissors and forces. The fine pieces of tissues were once again washed with PBS and drained. The tissues were transferred into sterile 250ml of conical flask having 50ml of 0.25% warm trypsin (temperature 37°C) and agitated mildly (@ 50rpm) for 15 minutes for trypsinization of parenchymal tissue. This trypsinization process was repeated one more time, and the first lot of disaggregated cells was discarded. The second lot of disaggregated cells suspension was filtered using sterile muslin cloth after neutralization of trypsin using FBS. Then filtered cell suspension was centrifuged at 1500 rpm for 10 minutes. The pellet was re-suspended in 5ml of MEM without FBS. Commercially available media formulation was used for the study. The harvested LT cells were grown in 25cc cell culture flask (Nunc™ EasYFlask™) in two type of media routinely used in the laboratory of CCSNIAH. Dulbecco modified eagles medium (DMEM) and Minimum Essential Medium with Earle's salt (MEM) supplemented with TPB and sodium bicarbonate was used

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Table1. Grouping of the flasks as per media composition and serum concentration

Flask no	Media	FBS supplement	Antibiotic +Anti-mycotic	TPB+Sodium bicarbonate
1	EMEM	5%	✓	✓
2	EMEM	10%	✓	✓
3	EMEM	15%	✓	✓
4	DMEM	5%	✓	✓
5	DMEM	10%	✓	✓
6	DMEM	15%	✓	✓

Table 2. Scoring of the flasks based on microscopic examination

Flask no	Sterility	Cell Senescence	Cell viability	Cell morphology
1	+++++	+	+	+
2	+++++	++	+	+
3	+++++	+	+++	+++
4	+++++	++	++	++
5	+++++	+	++++	++++
6	+++++	++	++++	++++

for the study. Fetal bovine serum (FBS) (Hi Media) from @ 5-15% was used. Antibiotic cocktail containing mixture of 10,000U/ml penicillin, 10 mg/ml streptomycin and 25µg/ml amphotericin B (Hi Media) was added to avoid microbial contaminations. MEM and DMEM supplemented with varying concentrations of FBS (5-15%) was used for seeding of harvested lamb testicles. About 200 µl of harvested lamb testicle cell suspension was added into 25 cc flasks having 5ml cell culture media as depicted in Table 1. All the flask were incubated in incubator at 37°C with 5% CO2. After 24 hr incubation, all the media from each flask was decanted, and fresh media in respective flask was added followed incubation for 5 days at 37°C with 5% CO_2.

The flasks were observed microscopically over 5-7 days and evaluated on basis of cell proliferation rate, morphological assessment and contamination control/ sterility check. Morphological scoring based on confluency and structural integrity. Cell profiler, an open-source software was used to analyze the images (Carpenter *et al.* 2006). The sterility of the cultures was analyzed with respect to freedom from visible bacterial and fungal contamination. The freedom from mycoplasma contamination was deduced through mycoplasma detection kit (Himedia).

The flasks were observed microscopically and scored based on confluency, structural integrity and cell senescence. There was considerable heterogeneity in the cell morphology at preliminary observation. The flasks were observed on the 5th day of incubation after changing media 24 hrs after incubation. The flask presented cells with two distinct morphology: initial fibroblast-like elongated cells and less elongated epithelial like morphology. The flasks were scored morphologically, cell viability and sterility. The detailed score is presented in Table 2.

The flask no 3, 5 and 6 showed considerably good cell viability nearing 60% while the flask no 1, 2 and 4 showed higher cell senescence. The flask with DMEM media supplemented with 10 and 15% serum showed good cell viability although the flask with the DMEM + 10 % serum concentration could be considered the most efficient for propagation of the LT cells (Fig.)

In a similar study, exhibiting effect of DMEM (low glucose) and RPMI-160 on dog periosteum derived cells, Wu *et al.* 2009 reported that the osteogenic differentiation of PDCs was enhanced in DMEM as compared with that in RPMI 1640. Similarly, Izham et al in (2018) reported DMEM: F12 supplemented with 15% hiFBS could serve as an optimized culture media for high proliferation rate of SH-SY5Y cells. Even though both DMEM and MEM with earls salt are considered to be versatile media for diverse mammalian cell types but the significantly higher concentrations of glucose, amino acids and vitamins in DMEM makes it a richer media than MEM. The results of the study do make sense citing the study of Jones *et al.* 1999 wherein high extracellular glucose had direct effect

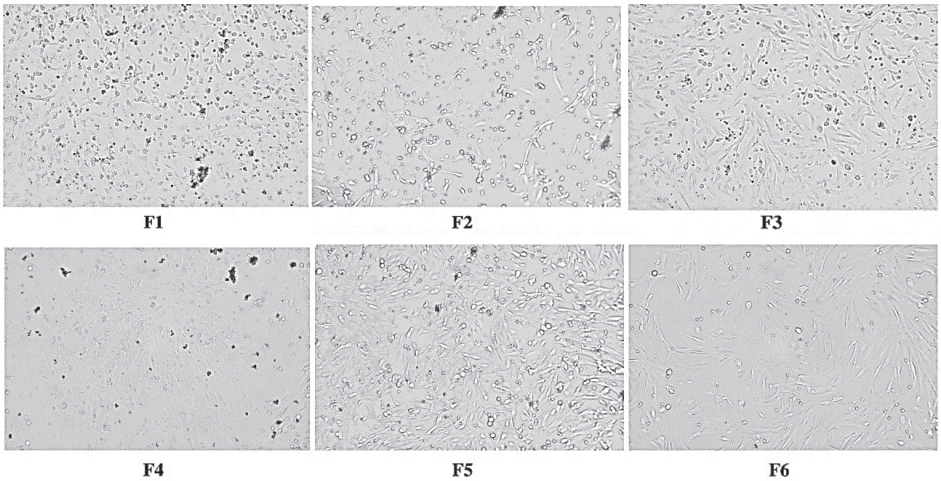


Fig. 1. Microscopic observation of the flasks (1-6) on day 5 post incubation

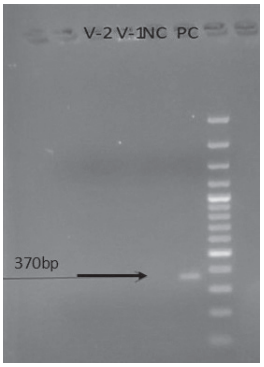


Fig. 2. PCR analysis of the flasks (V1 and V2) for mycoplasma detection with NC as Negative control and PC as positive control

on the cell growth and collagen synthesis of humans makes sense. However, certain studies have opinioned contrary to the result of the study by reporting decreased proliferation, differentiation, migration capacity, and mitochondrial activity in stem cells on exposure to high glucose micro environment (Tong *et al.* 2025). Nevertheless, it may be deduced that the effect of glucose may have varying effect on the varying cell types.

All the flasks were free of bacterial and fungal growth and samples from the flask were found to be negative for mycoplasma on PCR analysis. The sterility of the flasks was indicative of the efficiency of the antibiotics and the sterile procedural approach. Often cell culture techniques have been threatened by microbial contamination, which increases manifold while harvesting primary tissues from animals in abattoirs. The measures adopted during each step of harvest and propagation of the LT cells could be considered as ideal for achieving a sterile primary culture. Despite several inconveniences of primary cells such as, heavy preparation, heterogeneous cell population, non-reproducible viral titration and presence of potential endogenous contaminants, primary cells still provide excellent system for study of *in-vivo* viral dynamics.

SUMMARY

Primary cell culture is the *ex vivo* culture of cells freshly obtained from a multicellular organism, as opposed to the culture of immortalized cell lines. Primary cell cultures derived from testicular tissue serve as vital *in vitro* model for studying viral isolation and dynamics in wide range of viruses. Establishing stable primary testicular cell lines from lambs is often challenging due to variability in growth conditions, contamination risks, and lack of optimized culture media protocols. Selection of appropriate basal media and supplementation strategies plays a critical role in improving cell viability, proliferation, and maintenance of cellular morphology. However, there is limited comparative data on the efficiency of different media formulations for lamb testicular cell culture. LT cells are suitable for most of pox viruses, especially Lumpy Skin Disease virus. It may be noted that adapting a virus to primary cells yields more physiologically relevant results and more accurate model of *in vivo* infection for sequencing but researchers and vaccinologists are more conveniently dependent on the continuous cell lines for baseline studies and vaccine development. Hence, this study was designed to identify an optimized culture protocol for lamb testicular primary cell line preparation in two of the common cell culture media in varying serum concentration. Two commonly used laboratory media, DMEM and MEM with Earle's salt were used for the study. It was observed that DMEM supplemented with 10 % FBS could be an efficient and suitable medium for the propagation of the LT primary culture. The standardized protocol may help in the establishment of LT primary culture in the laboratory, understanding viral dynamics in the host, and evolutionary studies.

ACKNOWLEDGEMENT

The authors are grateful to The Director, CCSNIAH, Baghpat for his constant motivation. The authors are thankful to the Department of Animal Husbandry & Dairying, Govt of India for the support and cooperation.

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