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Isolation of bovine rotavirus in MDBK cell line from diarrhoeic calves of Navsari district

Pushpa M Makwana, IH Kalyani and Dhruv Desai

Abstract
The present study was designed to study isolation of rotavirus from faecal samples of bovine calves and to observe cytopathic effects of rotavirus. For this, total 157 faecal samples comprising 104 cattle and 53 buffalo calves were aseptically collected. Samples found positive by Latex Agglutination Test (LAT) were used for further study using MDBK cell line. By LAT 17 samples were found positive and these samples were used for cell culture study. All the 17 samples were inoculated in MDBK cell line and from these 16 samples could be successfully isolated, adapted and showed characteristic cytopathic effects. Out of 16 samples 4 samples showed cytopathic effect in first passage, 7 samples showed CPE after 2nd passage and 5 samples showed cytopathic effects at 4th passage level. The characteristic cytopathic effects includes rounding of cells, spindle shaped morphology and disruption of normal monolayer. The conformation of virus was checked by VP6 gene based reverse transcriptase PCR (RT-PCR).

Keywords: MDBK, CPE, cell culture, bovine rotavirus

Introduction
Among viruses, rotavirus is considered as one of the important agent for causing gastroenteritis and mortality in cattle and buffalo calves. Many infectious agents like bacteria, virus and protozoa are responsible for diarrhoeic condition in neonatal calves of varying species of animals and human infants, that is why definitive diagnosis is required for preventing infectious cause and proper treatment. For rotavirus diagnosis, agar gel immunodiffusion and counter- immunoelectrophoresis, complement fixation, radio-immunoassay, haemagglutination and haemagglutination inhibition, Latex agglutination test (LAT), RNA- Polyacrylamide gel electrophoresis (RNA-PAGE), Enzyme-linked immunosorbent assay (ELISA), Reverse transcription polymerase chain reaction (RT-PCR) and cell culture based virus isolation like different assays have been used by various research workers [1].
Rotavirus was first detected in cattle in the year of 1969 [2]. After that many workers have attempted isolation of rotavirus by cell culture technique. Though isolation of rotavirus using cell culture technique is a tedious and time consuming process, it gives the ultimate proof for the presence of virus. Isolation of bovine rotavirus (BRV) in the lab can be performed by using specific primary cell cultures (calf kidney cells) and cell lines (MA 104-Simian origin, MDBK and PK-15) [3].
Many workers have used MDBK cell lines for isolation and passage of group A rotaviruses from animals and humans with the use of Eagles MEM containing BSA, calf serum, antibiotics and fungicides [4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 1, 14, 3, 15].

Materials and methods
Cell line
MDBK cell line was procured from National Centre of Cell Science, Pune (India) and maintained in at Department of Veterinary Microbiology. Cell line was propagated in growth medium (GM) (Containing Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), non-essential amino acids (HyClone) and antibiotic antimycotic solution, Himedia). The cells were maintained in maintenance medium (DMEM supplemented with 2% FBS) during the study period.

Sub culturing of cells
The maintenance medium from the flask was decanted and cells were washed twice with sterile DPBS, after that 1 ml warm (temperature 37°C) filtered Trypsin Versene Glucose

Solution (TVGS) layered on monolayer with the help of 2 ml sterile plastic pipette with rubber bulb. TVGS was allowed to remain in to flask for 1- 2 minutes at 37°C, then medium was decanted and a film of TVGS was left in the flask. Detachment of cell monolayer could be visualized after slight shaking of flask and against the light. Then, 10 ml growth medium was poured into flask to inactivate the trypsin. Medium was repeatedly pipetted for 10-15 time with the help of 10 ml sterile disposable pipette with rubber bulb so that clumps of cells could be separated. From this flask, 5 ml cell suspension was transferred to fresh two T- 25 cell culture flasks. The flasks were incubated in CO_2 incubator at 37°C with 5% CO_2 concentrations and cells were allowed to form a monolayer sheet. Flasks were daily checked for pH of media and if there is change in colour found media was changed. By the same method repeatedly flasks were sub cultured in the ratio of 1: 3 till they were used for sample inoculation.

Preparation of fecal sample for inoculation / Passaging of rotavirus in cell line

A 10 per cent faecal suspension was prepared using 1X phosphate buffered saline (PBS, pH 7.4). After thorough vortex the suspension was clarified by centrifugation at 12000 g for 30 minutes in a refrigerated centrifuge (Heraeus Biofuge stratus centrifuge, Themosceintific) at 4ºC. The supernatant fluids were filtered through 0.45 µm membrane syringe filter and filtrates were mixed with an equal volume of Dulbecco's Minimum Essential Medium (DMEM) containing 2% fetal bovine serum (FBS) and 10 µg/ml crystalline trypsin and incubated at 37°C for 60 min. After incubation, one ml of the mixture was inoculated into the culture flasks with confluent monolayer of MDBK cell lines and kept for 1 hr incubation. After adsorption at 37°C for 1 hr, the cells were washed thrice with plain DMEM and then over layered with maintenance medium containing 1 µg /ml of crystalline trypsin and incubated at 37°C in CO_2 incubator [1]. One uninoculated flask was kept as a control.

Harvesting and propagation of the virus

The virus inoculated flasks were daily examined for cytopathic effects. Flasks that showed CPE in first passage and in subsequent passages were subjected to 3 cycles of alternative freezing and thawing. The cell culture fluid was collected in micro centrifuge tube and stored at -20°C for confirmation of virus.

Confirmation of virus in cell culture passaged samples

The cell culture fluid of the samples showed CPE were screened for the confirmation of rotavirus by VP6 gene-based RT-PCR. For this purpose, the cell culture fluids were subjected to RNA extraction using Quiagen viral mini RNA kit and then analyzed by RT-PCR.

Results and discussion

Total 17 samples were inoculated in MDBK cell line along with one uninoculated flask as a control. (Figure 1). Out of these, 16 samples were successfully adapted and showed characteristic cytopathic effects for rotavirus. Out of 16 samples, 4 samples showed cytopathic effect in first passage, 7 samples showed CPE after 2nd passage and 5 samples showed cytopathic effects in 4th passage level. The cytopathic effects included spindle shaped morphology, rounding of cells and disruption of normal monolayer which was started after 48 hours in first passage and after 24 hrs of second passage and fourth passage level (Figure 2 & Figure 3). In initial infection rounding of cells started in few cells that were visible after 24 hrs and 48 hrs in second and first passage respectively (Figure 3). After 72 hrs, clumping of cells was observed and more detachment of cells was seen (Figure 4, 5, 6). The cytopathic effects continued 96 hours p.i. with characteristics loss in cellular structures, all the cells became rounded and higher numbers of floating cells were observed (Figure 7). 120 hr p.i. (day 5) only few rounded cells were visible and most cells were found detached (Figure 8). After that complete destruction of cell sheet was observed.

After complete monolayer detachment, by freezing and thawing process the fluid was harvested and centrifuged at low speed and checked for confirmation of the virus by VP6 gene based RT- PCR.

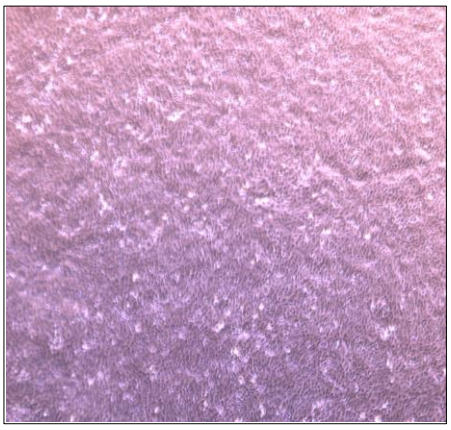

Fig 1: Normal MDBK cell line (4x)

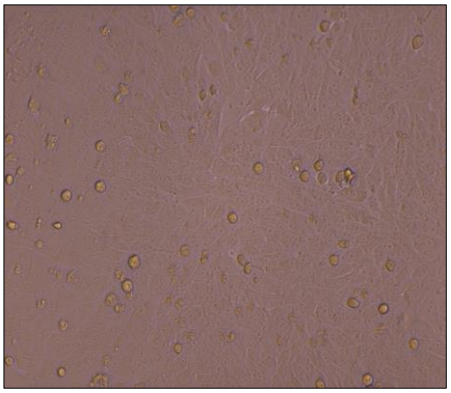

Fig 2: Initial stage of infection showing rounding of cells 24 hrs p.i. (20x)

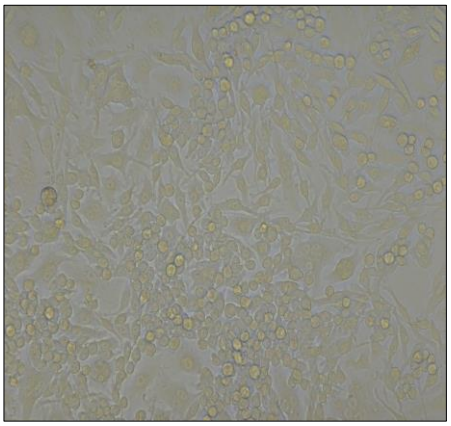

Fig 3: Infected cells showing more number of rounding cells 48 hrs. p.i. (20x)

Fig 4: Cellualar detachment, spindle shaped morphology and vacuolation 72 hrs. p.i. (20x)

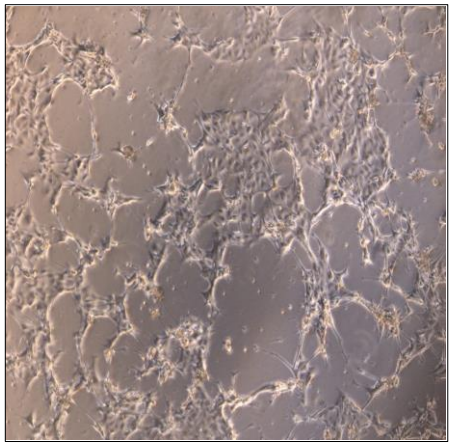

Fig 5: Cellular detachment and clumping of cells 72 hrs. p.i. (4x)

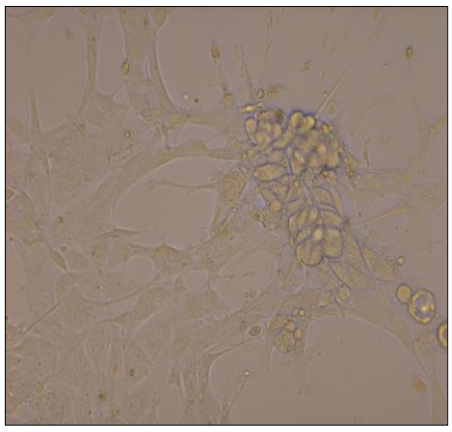

Fig 6: Clumping of cells and spindle shaped morphology 72 hrs. p.i. (40x)

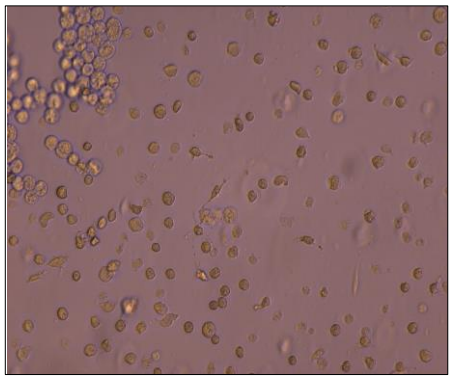

Fig 7: Most of the cells showing rounding of cells 96 hrs. p.i. (20x)

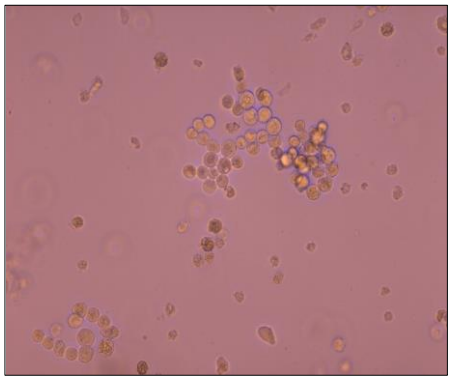

Fig 8: More cellular detachment and rounding of cells 120 hrs p.i. (20x)

These types of changes in MDBK cell line were typical of rotavirus infection. Similar to this, other workers [1] have studied isolation of rotavirus samples in MDBK cell line and observed characteristic cytopathic effects (CPE) from second passage i.e. clumping and rounding of cells, detachment of monolayer, intracytoplasmic eosinophilic inclusions, syncytia and leaving empty vacuole space in MDBK cell line. Others have [3] also observed characteristic cytopathic effects in 5 out of 50 diarrhoeic calve samples like rounding of cells, shrinkage of cell wall together with an increased granularity and progression to form a bunch of grapes within72-96 hrs. In the present study faecal samples were treated with trypsin and then samples were inoculated in the culture medium. Similar to this many workers have given the pre treatment of trypsin to the faecal samples that increases the infectivity of rotavirus. Similarly other workers [16] stated that incorporation of trypsin in the culture medium in small quantities enhance cytopathic effect (CPE) and also by the pretreatment of fecal samples with trypsin. One of the research worker [17] also suggested pre treatment of faecal inoculums with trypsin (5- 10 µg /ml), and 1.0 µg /ml trypsin in the maintenance medium for the continuous propagation of field isolates of bovine rotaviruses.

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