ORIGINAL PAPER

Cytopathic effect of Vero cells adapted Bangladeshi strain of peste des petits ruminants (PPR) virus in cell culture

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Abstract

The present study described the cytopathic effect of PPR virus presently being used in serial passages at the level of 60th in Vero cells and infected tissue culture fluid was used in this study as viral inoculum. Vero cells were grown on cover slip & were infected with tissue culture fluid at a fixed multiplicity of infection (MOI) 0.01. The infected cover slip along with control were stained with H&E stain at periodic intervals and cytopathic effect was studied with microscope. The cytopathic effect (CPE) was visible at first from 24 hpi and the Vero cells showed initial cell rounding, aggregation, and syncytial development. Development of inclusion bodies and cell degradation was noticed by 72 hpi. Complete detachment of the cell monolayer was observed by 84 hpi. It is concluded that, development of numerous inclusion bodies are the indication of well adaptation & extensive multiplication of PPRV in Vero cells.

Keywords Cytopathic effect (CPE) · Vero cells · Peste des petits ruminant's virus (PPRV) · H& E stain

Introduction

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterized by fever, oculonasal discharges, stomatitis, diarrhea and pneumonia (OIE 2004). The disease is caused by PPR virus belonging to the genus *Morbillivirus* in the family *Paramyxoviridae* (Gibbs et al 1979). Evidence of PPR in Bangladesh was reported for the first time by Sil et al (1995) and Bussell and Karzon (1965b).

Since then PPR is endemic in this country and few studies have been done to confirm the virus, but an intensive research in this context is not available. Virus production occurred several hours before the cytopathic effect could be detected in living cultures. The cytopathological study might show changes in the early stages of the cycle of virus multiplication in cultured cells (Reissig et al 1956; Bussell

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and Karzon 1962). The present paper reports such a study in which the intracellular changes occurring in monolayer culture of African green monkey kidney cells (Vero cells) after infection with PPR virus, has been related to the growth cycle of the virus. Therefore, the present study has been carried out to describe the cellular changes or alterations occurred in Vero cells after infection with peste des petits ruminants (PPR) virus presently circulating in the country.

Materials and methods

Materials

Cell line

Vero cells (CLS, Germany; order no. 605372) were brought from Germany and used in this study.

Cell culture media and reagents

Cell culture medium-M-1999 (Gibco-Invitrogen, cat no. 11825), calf serum-FBS (Gibco—Invitrogen, cat no. 10437), 0.25% Trypsin with EDTA (Gibco-Life technologies 20367, C13) were used in this study.

Viral inoculum

Locally isolated PPR virus (BD/PPR/08) was serially passaged in Vero cells from 1st passage up to 60th Passage for the purpose of adaptation and attenuation. Infected tissue culture fluid at 60th passaged level was used in this study as viral inoculum.

PPR viral RNA confirmation

For the confirmation of presence of viral RNA in tissue culture fluid RT-PCR was done in each five (05) passage interval. Only after confirmation of presence of viral RNA at 60th passaged level, TCF is used in this study as viral inoculum.

Methods

Growth of Vero cells on coverslips and infection

Vero cells were grown on 40×25 mm coverslip in 50 mm diameter Petri dish. Total 24 Petri dishes were used for this
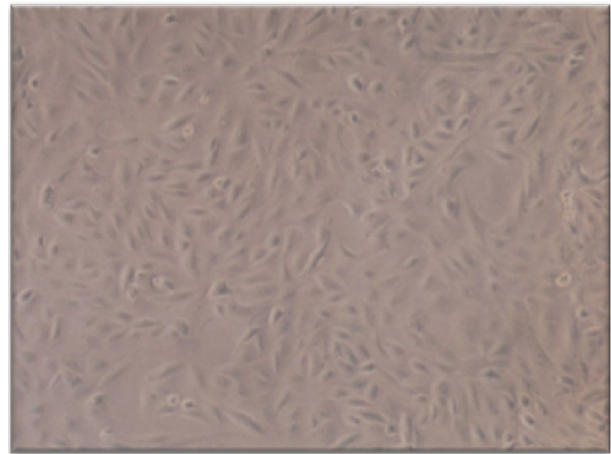

Fig. 1 Confluent monolayer of Vero cells, $\times 82.5$, phase contrast

study. When the cells become confluent on coverslip, the growth medium was removed with a pipette and the cell sheets on the cover slips in 12 Petri dishes were inoculated with 100 µl of PPR virus (BD/PPR/08) infected tissue culture fluid of 60th passage (titre 6.5 log10 TCID$_{50}$/ml) at a fixed multiplicity of infection (MOI) of 0.01. For MOI calculation the TCF was titrated following end point dilution assay (Reed and Muench 1938) and the number of cells were calculated by automated cell counter. After 1 h incubation, 4 ml of maintenance medium with 5% Newborn calf serum was added and the Petri dishes were incubated as before. Without virus inoculation, only media was changed for rest 12 Petri dishes. These Petri dishes were treated as a control. Two infected coverslip and two controls were sampled at each of the six time groups (24, 36, 48, 60, 72 and 84 hpi).

Staining of infected coverslips

Infected coverslips were stained with H&E stain as described by Titford (2009) and Barski et al. (1955). Briefly, the cover slip containing cell sheet was rinsed in PBS and fixed in ice-cold methanol for 30 min. After fixation, cover slip was transferred to 80% alcohol and changed several times. Then the cells were stained overnight in diluted Harris hematoxylin (20–30 drops of Harris hematoxylin in 100 ml water). Then the cover slips were washed in running tap water for 15 min and stained with eosin for 1–3 min. The coverslips were differentiated and dehydrated in several changes of 95% and absolute alcohol. Finally, the cover slips were cleaned in xylene and mounted on slides using Dibutylphthalate polystyrene xylene (DPX) with the cell sheets upside down. The stained coverslips were examined under microscope to observe the cytopathology of the PPR virus.
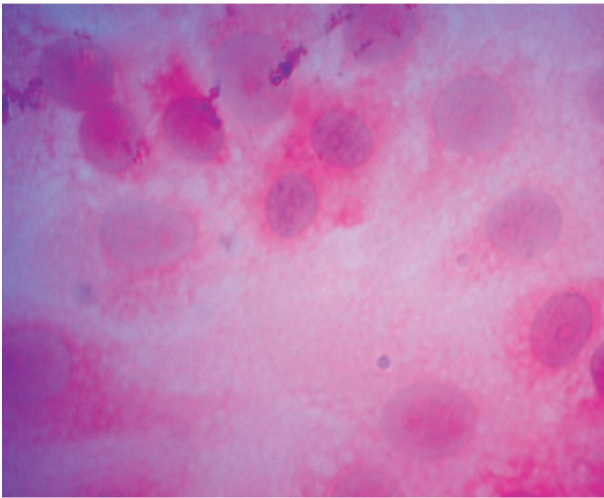
Fig. 2 Normal Vero cells at 12 h post seeding, H & E, ×100

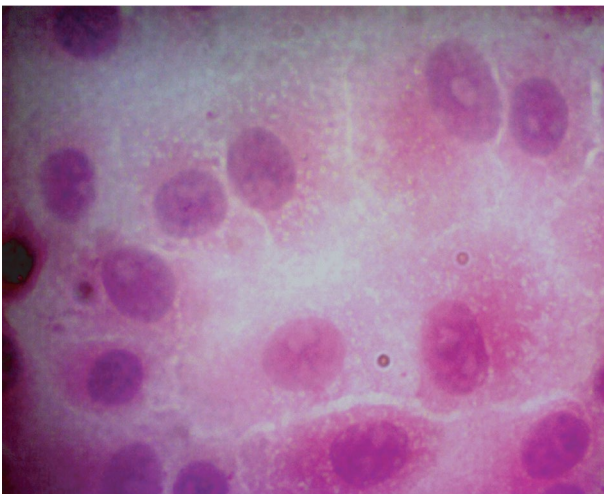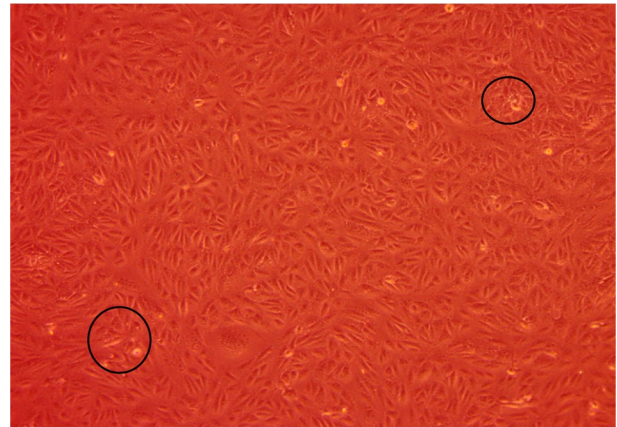Fig. 4 CPE (*circled areas*) on 12 hpi at 60th passage level, initial stage of cell rounding, ×82.5, phase contrast
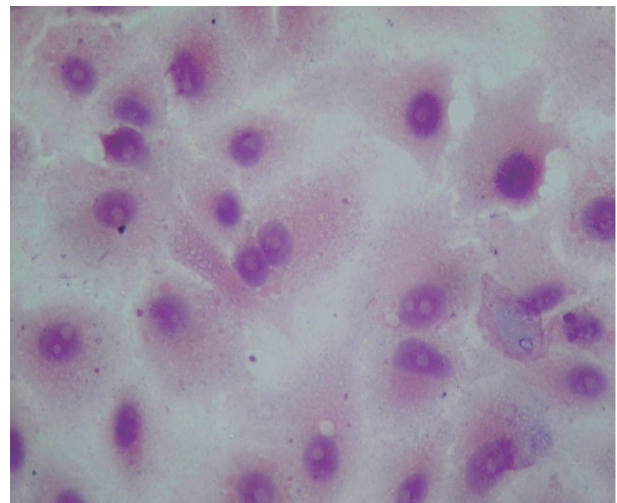

Fig. 3 Normal Vero cells at 36 h post seeding, H & E, ×100

Fig. 5 PPRV infected monolayer: shows apparently normal Vero cells at 12 hpi. Initial cell rounding observed. H&E, ×40

Results

Vero cells of control group

Confluent monolayer of normal Vero cells at both unstained and stained (H&E stain) on 12 and 36 h post seeding (Fig. 1, 2, 3) conditions are also shown for comparative study with same time category infected cells. Cells of all non-infected controls showed almost similar morphology in microscopic study. The cells were flattened, disc or fish scale shaped with almost rounded nuclei.

Morphology of cytopathic effect (CPE) in Vero cells by adapted PPRV isolates

The morphology of cytopathic effect (CPE) at 60th passage label includes detachment, cell rounding and aggregation of cells or cell clustering i.e. syncytia formation which were very resemblance to the morphology of cytopathic effect (CPE) produced at earlier passages of infection. In early stages, cell rounding does not appear within 12 hpi but at 60th passage label, initial stage of cell rounding appeared at 12 hpi (Figs. 4, 5). As infection progressed, the cells become
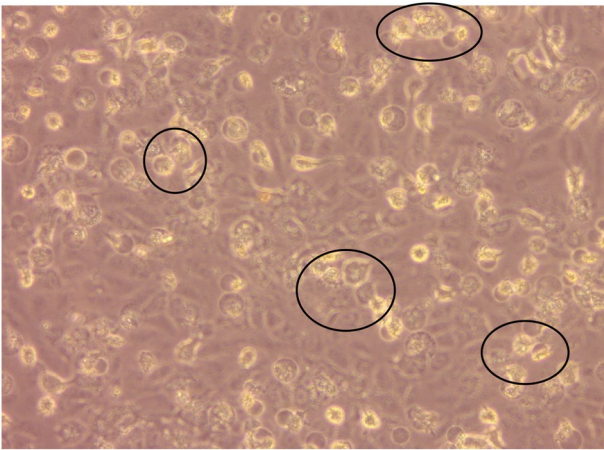
Fig. 6 CPE (*circled areas*) on 36 hpi at 60th passage level: cell rounding and numerous small syncytia (2–3 cell) formation, ×82.5, phase contrast
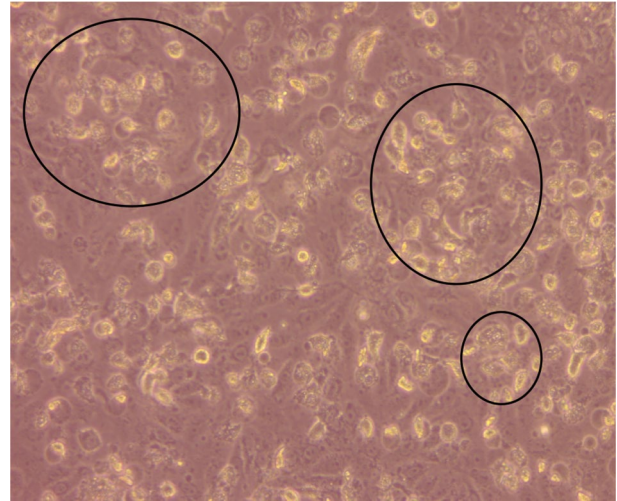

Fig. 8 CPE (*circled areas*) on 48 hpi at 60th passage level: large and small syncytia, ×82.5),phase contrast
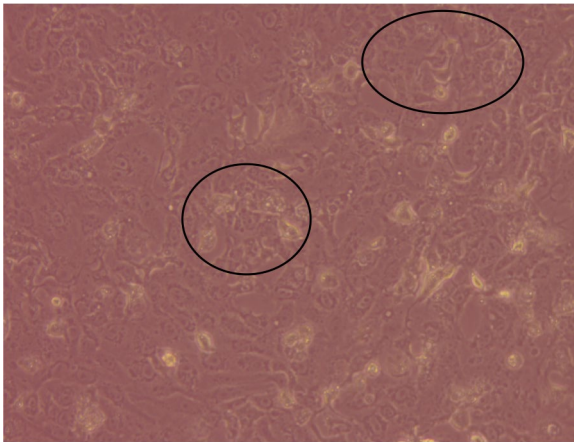

Fig. 7 CPE (*circled areas*) on 24 hpi at 60th passage level: cell rounding and clustering, ×82.5, phase contrast
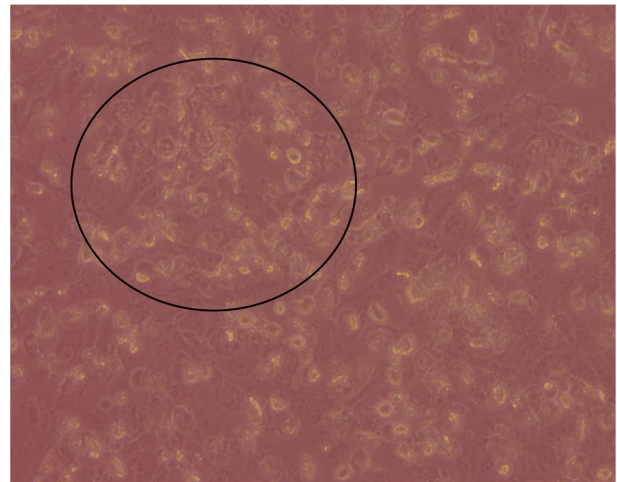

Fig. 9 CPE (*circled area*) on 60 hpi at 60th passage level: large syncytia, ×82.5, phase contrast

smaller; rounded, showed a thin peripheral layer of cytoplasm (Fig. 4). The affected cells fused to form small group or cluster or syncytia (Fig. 6) which in later transformed into large syncytia (Figs. , 6, 78, 9) and finally the monolayer of cells were completely detached from floor of the cell culture flask (Figs. 10).

CPE produced in Vero cells on cover slip (stained with H&E) by PPRV

Vero cells of infected group: cytoplasmic changes

Initially, the alterations in the cytoplasm began at 24 hpi, includes a single, well defined, eosinophilic cytoplasmic mass adjacent to the nucleus (Fig. 11). The cytoplasmic fusion among adjacent cells happened, results formation of

small syncytia consisting of approximately 3 cells (Fig. 11). As the infection progressed, the number of formation of small syncytia increased up to 60 hpi. Gradually, the syncytia increased in size, consisted of around 5 cells at 36 hpi (Fig. 12), around 10 cells at 48 hpi (Fig. 13), around 15 cells at 60 hpi (Fig. 14). Numerous deeply stained eosinophilic cytoplasmic granules, termed as intracytoplasmic inclusion bodies varied in diameter and number were seen in cytoplasm of cell (Fig. 15). In earlier study with BD_ PPR_2008, less intracytoplasmic inclusion bodies were seen at 72 hpi on 9th passage level (Fig. 16, collected from PhD Thesis, Rahman 2013) but on 60th passage level more inclusion bodies (varied between 6 and 8) were seen at same time category (Fig. 15).

Fig. 10 CPE (*circled area*) on 72 hpi at 60th passage level: almost complete detachment of cell monolayer, ×82.5, phase contrast

Fig. 12 PPRV infected monolayer: small syncytia formation (around 5 cells-circled area) on 36 hpi at 60th passage level. H & E, ×100

Fig. 11 PPRV infected monolayer: small syncytia formation (around 3 cells-circled area) on 24 hpi at 60th passage level. H&E, ×100
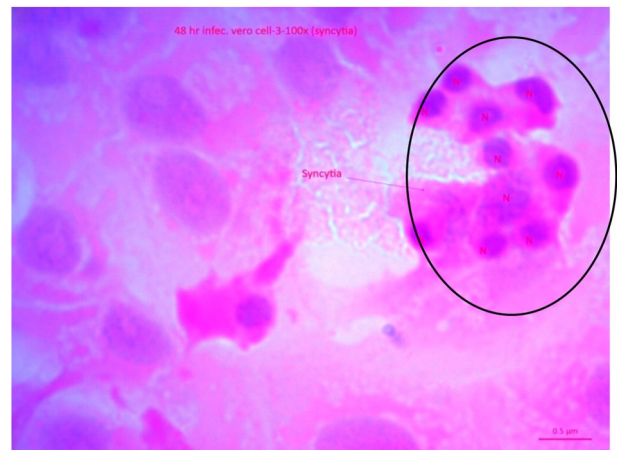

Fig. 13 PPRV infected monolayer: large syncytia formation (around 10 cells-circled area) on 48 hpi at 60th passage level. H &E, ×40

Vero cells of infected group: nuclear changes

A significant nuclear alteration at 60th passage level appeared at 36 hpi. The inclusion bodies called intranuclear inclusion bodies were developed and it was found that, the number of intranuclear inclusion bodies increased up to 60 hpi and thereafter maintained a steady state level. The intranuclear inclusion bodies were found as small eosinophilic granules, more in number initially but become larger in size in later stage, though number reduced gradually (Figs. 17, 18). The number of small granules varied from 1 to 7 per nucleus but in later stages, only one or two large granules developed per nucleus, and

these were more round and deeply eosinophilic (Figs. 18, 19). In very later stage (72 hpi) degradation of cells were observed (Fig. 19).

Discussion

Qualitative observations indicated that, a certain sequence of alterations or changes occurred on Vero cells as the cytopathic effects of PPR virus. In the present study, the following cytopathic features were prominent: cell rounding's, syncytia formation, eosinophilic nuclear granules (intranuclear inclusion body), cytoplasmic mass formation, and basophilic cytoplasmic granules (intracytoplasmic inclusion bodies). Similar CPE is observed on fourth day post infection (4 dpi) with Sungri strain and on 36–48
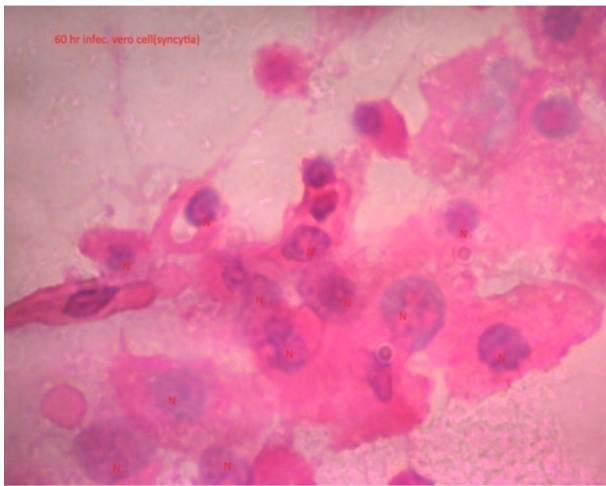
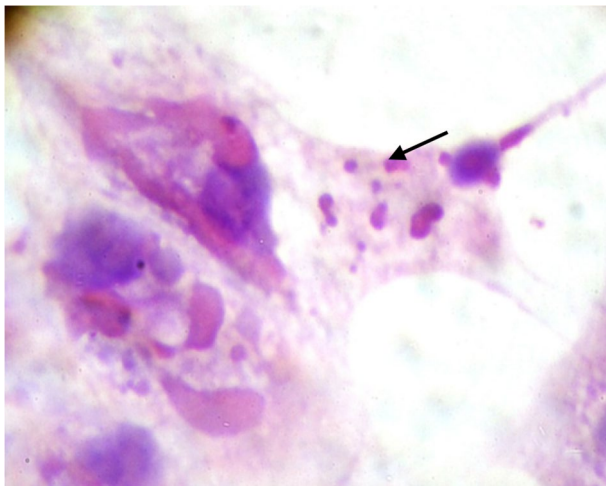**Fig. 14** PPRV infected monolayer: large syncytia formation on 60 hpi at 60th passage level. H & E, ×40

Fig. 16 Intracytoplasmic inclusion bodies (*arrow*) in Vero cells found on 72 hpi at 9th passage level. H &E, X 100 (Courtesy: PhD Thesis, Rahman 2013; ref-33)

Fig. 15 PPRV infected monolayer: several intracytoplasmic inclusion bodies on 72 hpi at 60th passage level. H &E, ×100

Fig. 17 PPRV infected monolayer: intranuclear inclusion body; larger granules found (*circled area*) on 60 hpi at 60th passage level. H & E, ×100

hpi with Arasur strain of PPR vaccine virus in Vero cells (Hegde et al. 2009). The results were also in accordance with earlier reports of Lefevre and Diallo 1990, Mohan (2004) in Vero cell, John et al. (2006) in BHK-21 cells, and Sreenivasa et al. (2006) in Marmoset B95a cells. Cytoplasm exhibited vacuolization, membrane fusion resulting formation of small syncytia in early stages and large syncytia in later were in agreement with other paramyxoviruses such as canine distemper virus in Vero cells (Bussell and Karzon 1965a. 1965b; Rankin et al. 1972; Guo and Lu 2000; Seki et al. 2003; Nishi et al. 2004; Lan et al. 2005) in hamster cells (Serageldeen et al. 2009), dog and bovine kidney cell (Bussell and Karzon 1965a), in ferret embryonic lung cells (Rankin et al. 1972), in chic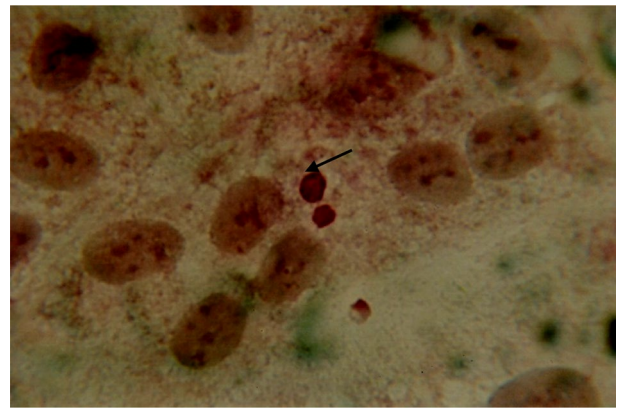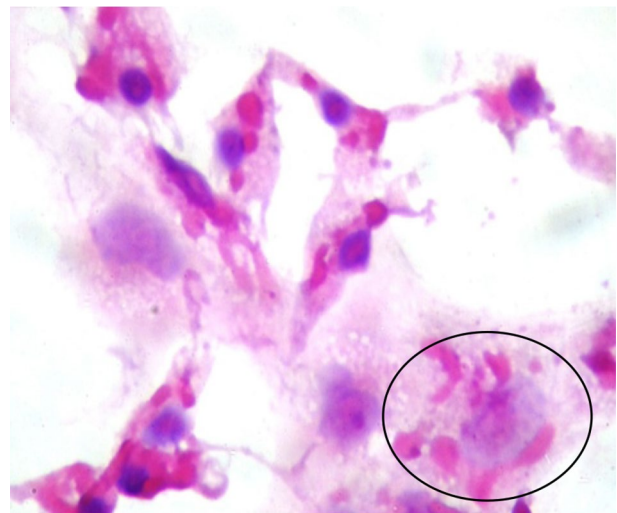k embryo cell culture (Bussell and Karzon 1962), Rinderpest virus (RPV-Egypt- and RPV-Saudi strain) in bovine monocyte cells (Nores and McCullough 1997), in Vero cells (Mahapatra et al. 2006), Measles virus (MVwtD4, MVwtD8, and MVwtH1 strain) in Vero/hSLAM cells, Sendai paramyxo virus in MDBK (Madin-Darby bovine kidney cells) cells (Scheid and Choppin 1974). Dunnebacke (1956) and Buckley (1957) studied the effect of all three types of Polio virus on Vero cells, HeLa, Human fetal and Human amnion cells, measured the cytopathic effects by light microscopy of stained cells and divided the events or alteration in to four stages (1) early nuclear pyknosis and certain cytoplasmic changes (2) more advanced changes (3) accumulation of nuclear masses and (4) finally cell disintegration which almost supported the present findings.
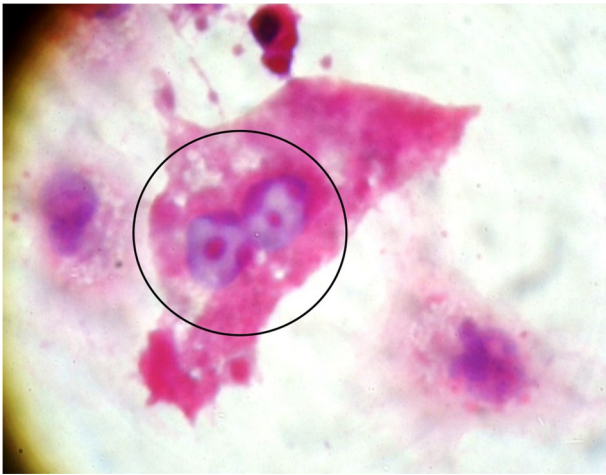
Fig. 18 PPRV infected monolayer: deeply stained round large eosinophilic IB (*circled area*) on 72 hpi at 60th passage level. H & E, ×100
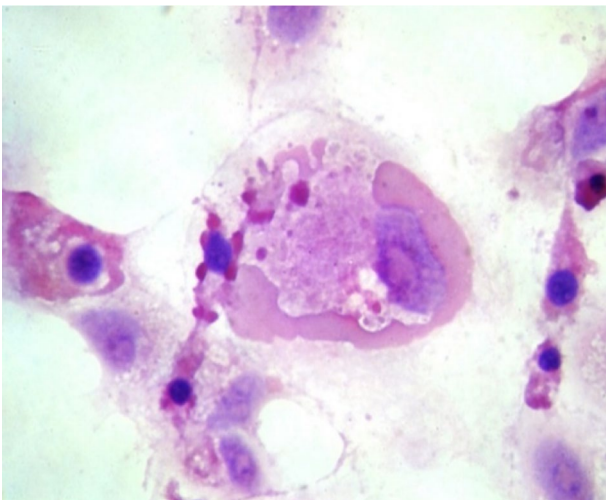

Fig. 19 PPRV infected monolayer; degradation of cells at very late stage (72 hpi). H & E, ×100

Infected coverslip cultures stained with H&E stain showed intracytoplasmic and intranuclear inclusions or granules, varied in number with time category in this study, which is supported by Hegde et al. (2009) and Mohan (2004) in Vero cells by PPRV, Pereira (1958) in HeLa cells by adeno virus. Differences in cytopathology in Vero cells inoculated with virulent and attenuated CDV strains have been reported by Confer et al. (1975). After that, the virulent R252 and Snyder Hill strains were adapted to grow in Vero cells by sub passaging 5 times, it is found that, the strains produced eosinophilic nuclear inclusions on 7 days post infection (pi) in Vero cells stained with May-Grunewald Giemsa stain. Distinct fluorescent nuclear bodies were also seen with the virulent strains in cells stained 7 days post infection with fluorescein-labeled CDV antibody. The only attenuated strain examined was the Onderstepoort strain, and although it produced polykaryocytes and exhibited cytoplasmic fluorescence that was typical of the virulent strains, it did not produce nuclear inclusions or fluorescing nuclear bodies. Nuclear aggregates were also not observed when the cells were examined with electron microscopy. Other authors have described a similar progression of events in a human fibroblasts single cell by poliomyelitis virus studied under phase-contrast and bright-light microscopy by Lwoff et al. (1955) and Barski et al. (1955). Similar cellular alterations also were reported by Harding et al. (1956) in the living state as well as in fixed and stained preparations of HeLa cells by the same virus. The virus isolate (BD_PPR_08) produced both intranuclear and intracytoplasmic inclusion body on 72 hpi at both 9th passage by Rahman et al. (2011) and 60th passage level in this study, but the difference is the amount of inclusions body is much more higher at 60th passage level than earlier which indicates more adaptation. This is also in accordance with the development of inclusions in tissue cultures of monkey kidney epithelial cells infected with poliomyelitis studied by Beale et al. (1956). Detachment from glass surface and cell rounding or clumping were observed in this study at 24 hpi which was similar to the findings of Rowe et al. (1958) in Hela cells or KB cells monolayers by adenoma virus.

Conclusion

It is concluded that, the development of a sequence of cellular changes (CPE) is the indication of well adaptation of PPR virus in Vero cells and once adapted, virus multiplied extensively in Vero cells, which is very important in potent vaccine production strategy.

Authors' contributions This research was conducted under the direct supervision of the two co-author (Prof. Dr. Emdadul Haque Chowdhury & Prof. Dr. Md. Rafiqul Islam) where they played role as Supervisor and Co-supervisor respectively of the PhD research of the corresponding author (Md. Saiful Islam Siddiqui). MR Islam and EH Chowdhury

Availability of data and materials Available on request.

Code availability N/A.

Declarations

Conflict of interest No conflict of interest.

Ethics approval N/A.

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