Cytopathic Effect of Vero Cells Adapted Bangladeshi Strain of Peste Des Petits Ruminants (PPR) Virus in Cell Culture

MSI Siddiqui (msisiddiqui2000@yahoo.com)
Sylhet Agricultural University

Anja Globig
Friedrich Loffler Institut, Federal Research Institute for Animal Health

Bernd Hoffmann
Friedrich Loffler Institut, Federal Research Institute for Animal Health

MN Islam
Department of Livestock Services, Ministry of Livestock and Fisheries

MR Islam
Bangladesh Agricultural University

EH Chowdhury
Bangladesh Agricultural University

Research Article

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Abstract

The present study described the cytopathic effect of PPR virus presently being used in serial passages at level of 60^{th} 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 is the indication of well adaptation & extensive multiplication of PPRV in Vero cells.

Highlights

- Locally isolated Bangladeshi strain of *peste des petits ruminants* (PPR) virus adapted well in Vero cells
- The cytopathic effect (CPE) was visible at first from 24-hour of post infection (hpi) and the Vero cells showed a sequence of changes includes - initial cell rounding, aggregation and syncytial development. The generalized CPE including large syncytia formation, development of more inclusion bodies.
- Complete cell degradation was noticed by 72 hrs post infection (hpi).
- However complete detachment of the cell monolayer was observed by 84 hrs post infection.
- Numerous acidophilic intracytoplasmic and intranuclear inclusion bodies indicated that the virus multiplied extensively in Vero cell.

Introduction

Peste des petits ruminants (PPR) is an acute viral disease of small ruminants characterized by fever, oculonasal discharges, stomatitis, diarrhea and pneumonia [1] . The disease is caused by PPR virus belonging to the genus *Morbillivirus* in the family *Paramyxoviridae* [2]. Evidence of PPR in Bangladesh was reported for the first time by Sil et al. [3] . 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 [4]. The present paper reports such a study in which the intracellular changes occurring in monolayer culture of monkey kidney 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 and Vero cells adapted by serial passaging (60^{th} passaged level) PPR virus (BD/PPR/08) infected tissue culture fluid was used in this study as viral inoculum.

PPR viral RNA confirmation

Tissue culture fluid only after confirmation of presence of viral RNA by RT-PCR was used in this study as viral inoculum.

Methods

Growth of Vero cells on Coverslips & infection

Vero cells were grown on 40 x 25 mm coverslip in 50 mm diameter Petri dish. Total 24 Petri dishes were used for this 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 60^{th} passage (titre 6.5 log10 $TCID_{50}$/ml) at a fixed multiplicity of infection (MOI) of 0.01. After one hour 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 84hpi).

Staining of infected coverslips

Infected coverslips were stained with H & E stain described by Titford [5] . Briefly, the cover slip containing cell sheet was rinsed in PBS and fixed in ice-cold methanol for 30 minutes. After fixation, cover slip was

transferred to 80 % alcohol and changed several times. Then the cells was 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 minutes and stained with eosin for 1-3 minutes. 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.

Results

Vero cells of control group

Confluent monolayer of normal Vero cells at both unstained and stained (H& E stain) on 12 and 36 hr 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

In general, the cells become contracted and decrease in size over the time. Initial stage of cell rounding appeared at 12 hpi (Fig. 4 &10). As infection progressed, the cells become smaller; rounded, showed a thin peripheral layer of cytoplasm (Fig. 4). Detachment, rounding and aggregation of cells, at 24 hpi (Fig. 5) and at 36 hpi (Fig. 6). The affected cells fused to form small group or cluster or syncytia (Fig. 6) which in later transformed into large syncytia (Fig. 7 and 8) and finally the monolayer of cells were completely detached from floor of the cell culture flask (Fig. 9).

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

Vero cells of infected group:Cytoplasmic changes

Alterations in the cytoplasm began at 24 hpi. Initially, the cells showed a single, well defined, eosinophilic cytoplasmic mass adjacent to the nucleus (Fig. 11). The mass frequently displaced the nucleus to one side of the cell, and the cytoplasm of few adjacent cells come in contact (cytoplasmic fusion) and gathered together to form small syncytia consisting of approximately 3 cells (Fig. 11). As the infection progressed, the number of small syncytia increases up to 60 hpi. The syncytia increased in size with time and 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 varied in diameter and number were seen in cytoplasm of cell, termed as intracytoplasmic inclusion bodies (Fig. 15). In earlier study with BD_ PPR_2008, very few intracytoplasmic inclusion body was found at 72 hpi on 9^{th} passage level (Fig. 19, collected from PhD Thesis, Rahman MM, 2013) but on 60^{th} passage level the inclusion body increased in number and number varied between 6-8 at same time category (Fig. 15).

Vero cells of infected group: Nuclear Changes

A significant nuclear alteration at 60th passage level appeared at 36 hpi. The number of nuclei containing eosinophilic granules (inclusion body) was increased up to 60 hpi and thereafter maintained a steady state level. The eosinophilic granules were smaller in size and increased in number at the beginning of its appearance but at later stages the size of the inclusion became larger and number also reduced (Fig. 16 – 17). The number of small granules varied from 1 to 7 per nucleus, termed as intranuclear inclusion bodies. In the later stages, only one or two large granules per nucleus were present, and these were more round and deeply eosinophilic (Fig. 17 and 18). In very later stage (72 hpi) degradation of cells were observed (Fig. 18).

Discussion

Qualitative observations of the cytopathic effects of PPR virus on cultures of African Green monkey kidney cells (Vero cells) indicated that a certain sequence of changes occurred. In the present study, the following cytopathic features were prominent: cell rounding's, syncytia formation, eosinophilic nuclear granules (intra nuclear inclusion body), cytoplasmic mass formation, and basophilic cytoplasmic granules (intracytoplasmic inclusion bodies). Similar CPE was observed on fourth day post infection with Sungri strain and on 36-48 hpi with Arasur strain of PPR vaccine virus in Vero cells [6]. The results were also in accordance with earlier reports of Lefevre and Diallo [7] , Mohan [8] in Vero cell, John *et al.* [9] in BHK-21 cells, and Sreenivasa *et al.* [10] in Marmoset B95a cells.

Cytoplasm exhibited vacuolization, membrane fusion resulting in small syncytia formation in early stages and in later large syncytia are in agreement with other paramyxoviruses such as canine distemper virus in Vero cells [11, 12, 13, 14, 15, 16,17], in hamster cells [18] , dog and bovine kidney cell [11] , in ferret embryonic lung cells [13], in chick embryo cell culture [19] , Rinderpest virus (RPV-Egypt- and RPV-Saudi strain) in bovine monocyte cells [20] , in Vero cells [21], Measles virus (MVwtD4, MVwtD8, and MVwtH1 strain) in Vero/hSLAM cells, Sendai paramyxo virus in MDBK (Madin -Darby bovine kidney cells) cells [22]. Dunnebacke [23] & Buckley [24] studied the effect of all three types of Polio virus on Monkey kidney (Vero), 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 (i) early nuclear pyknosis and certain cytoplasmic changes (ii) more advanced changes (iii) accumulation of nuclear masses and (iv) finally cell disintegration which almost supported the present findings. 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 were supported by Hegde and Mohan [6,8]) in Vero cells by PPRV, Pereira [25] in HeLa cells by adeno virus. Differences in cytopathology in Vero cells inoculated with virulent and attenuated CDV strains have been reported by [26] . After the virulent R252 and Snyder Hill strains were adapted to grow in Vero cells by sub passaging 5 times, the strains produced eosinophilic nuclear inclusions 7 days post infection 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 [27, 28]. Similar cellular alterations also were reported by Harding [29] 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 9^{th} passage [30] and 60^{th} passage level in this study, but the difference is the amount of inclusions body is much more higher at 60^{th} passage level than earlier which indicates more adaptation. This also in accordance with the development of inclusions in tissue cultures of monkey kidney epithelial cells infected with poliomyelitis studied by [31]. 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 [32] 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 cell, which is very important in potent vaccine production strategy.

Declarations

Acknowledgement

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Availability of data and material: Available on request

Code availability: N/A

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 conceived of the presented idea, verified the analytical methods and supervised the findings of this work. Anja Globig and Bernd Hoffmann (FLI, Germany) contributed to the enrichment of laboratory, sample preparation and transported sample from Bangladesh to FLI, Germany and carried out realtime RT-PCR and sequenced. Md. Nazrul Islam is another PhD fellow who supported the research work and designed the model and the computational framework and analysed the data. All authors discussed the results and contributed to the final manuscript. MN Islam and MSI Siddiqui wrote the manuscript with support from MR Islam and EH Chowdhury.

Ethics approval: N/A

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Figures

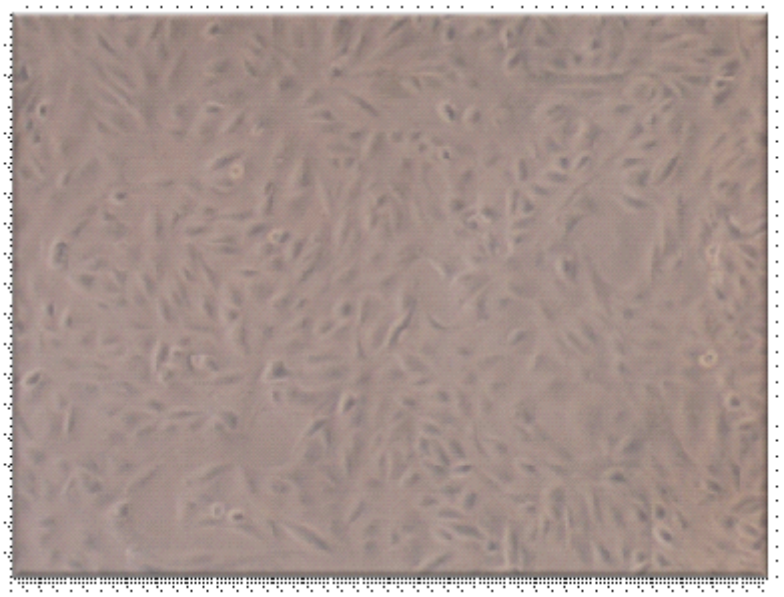

Figure 1

Confluent monolayer of Vero cells, x 82.5, phase contrast

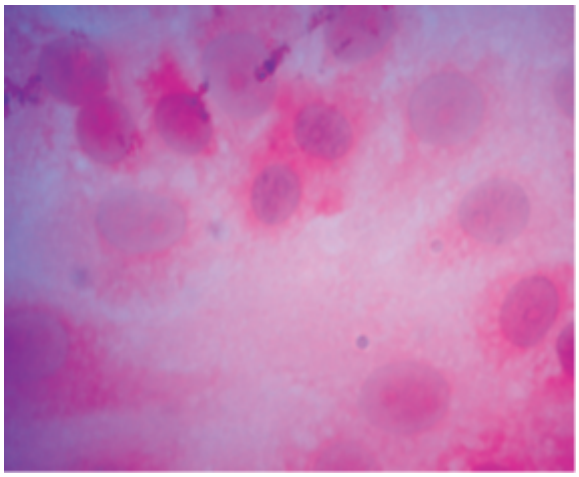

Figure 2

Normal Vero cells at 12 hr post seeding, H & E, x 100

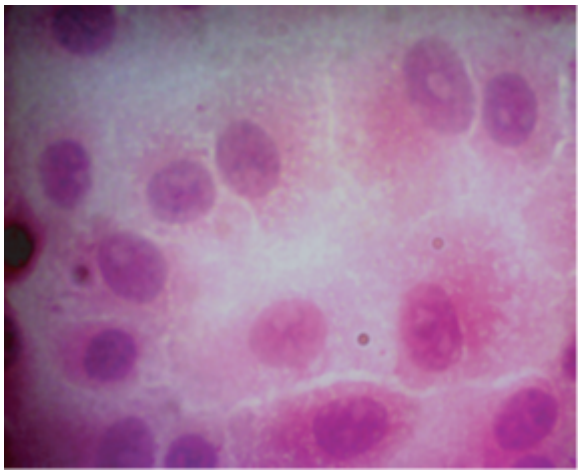

Figure 3

Normal Vero cells at 36 hr post seeding, H & E, x 100

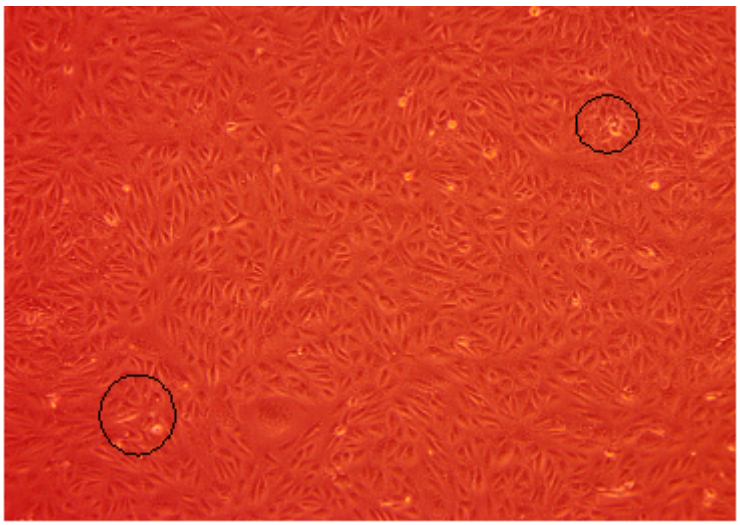

Figure 4

CPE (circled areas) on 12 hpi at 60th passage level, initial stage of cell rounding, x 82.5, phase contrast

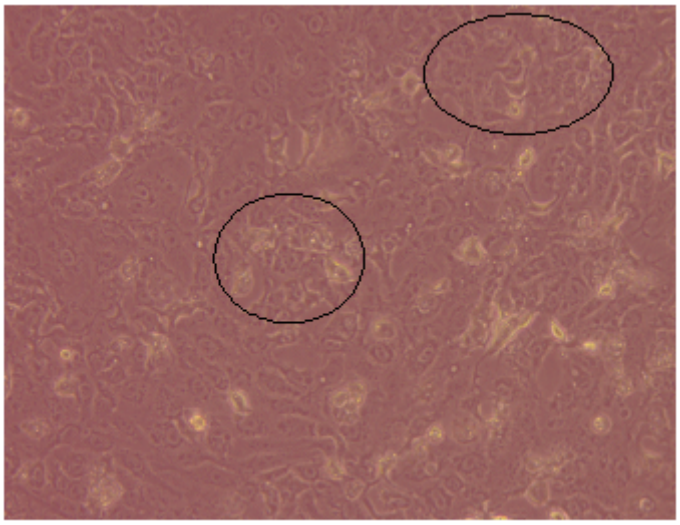

Figure 5

CPE (circled areas) on 24 hpi at 60th passage level: cell rounding and clustering, x 82.5, phase contrast

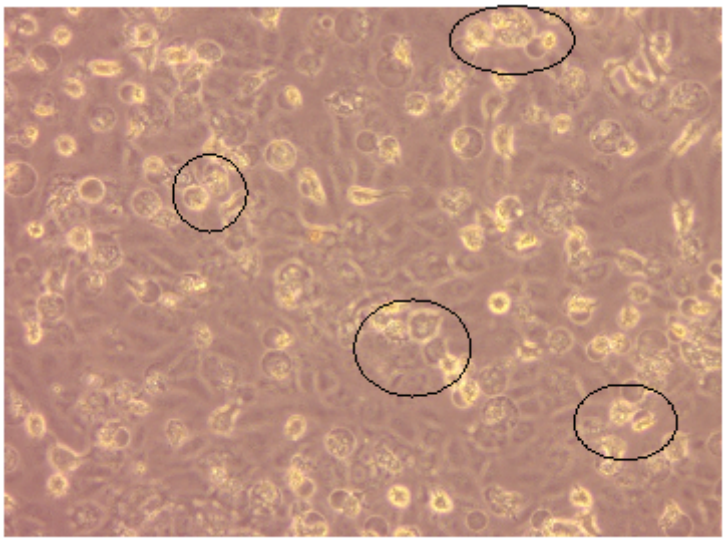

Figure 6

CPE (circled areas) on 36 hpi at 60th passage level: cell rounding and numerous small syncytia (2-3 cell) formation, x 82.5, phase contrast

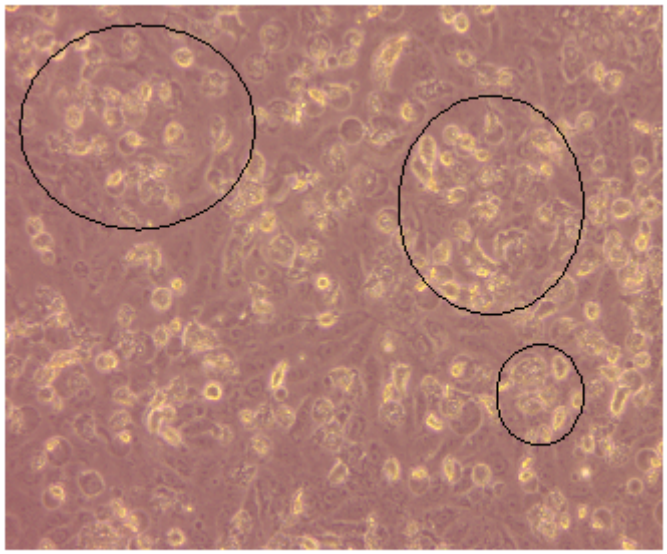

Figure 7

CPE (circled areas) on 48 hpi at 60th passage level: large and small syncytia, x 82.5),phase contrast

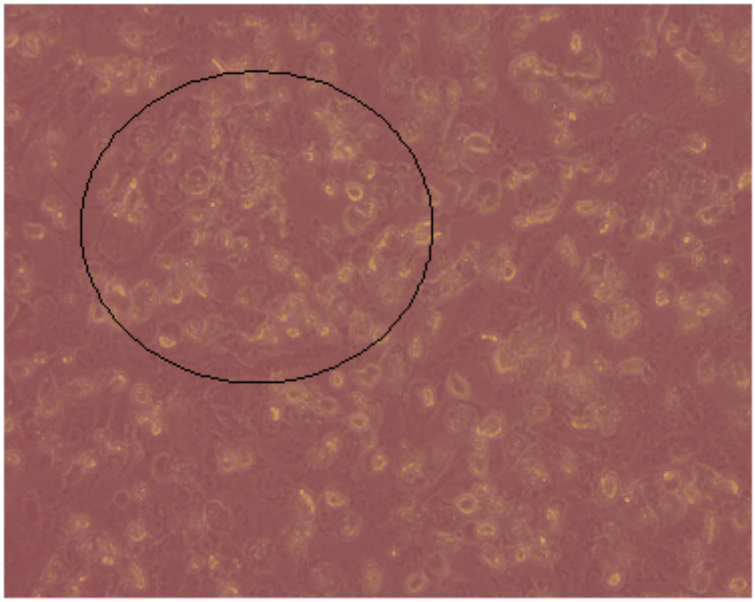

Figure 8

CPE (circled area) on 60 hpi at 60th passage level: large syncytia, x 82.5, phase contrast

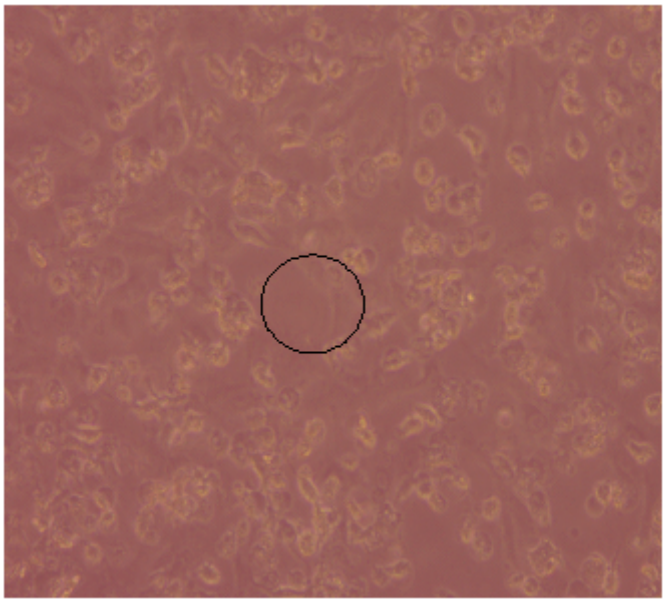

Figure 9

CPE (circled area) on 72 hpi at 60th passage level: Almost complete detachment of cell monolayer, x 82.5, phase contrast

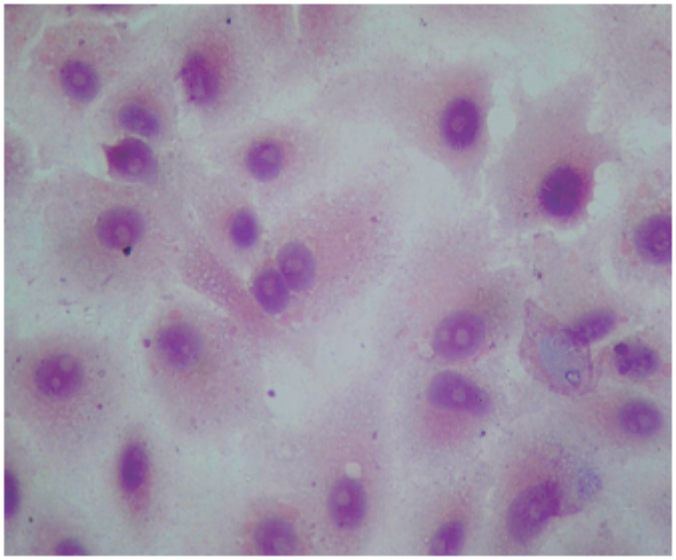

Figure 10

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

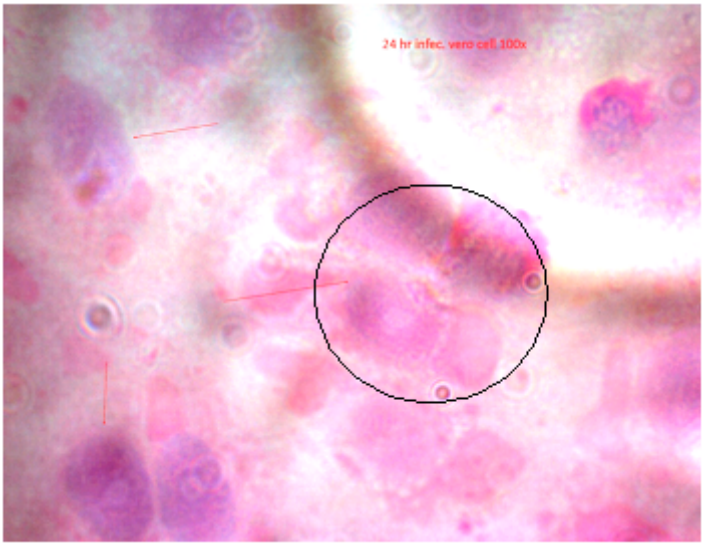

Figure 11

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

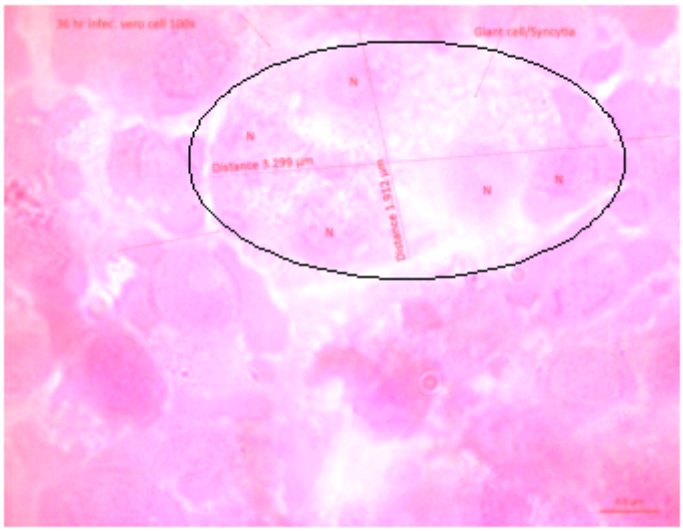

Figure 12

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

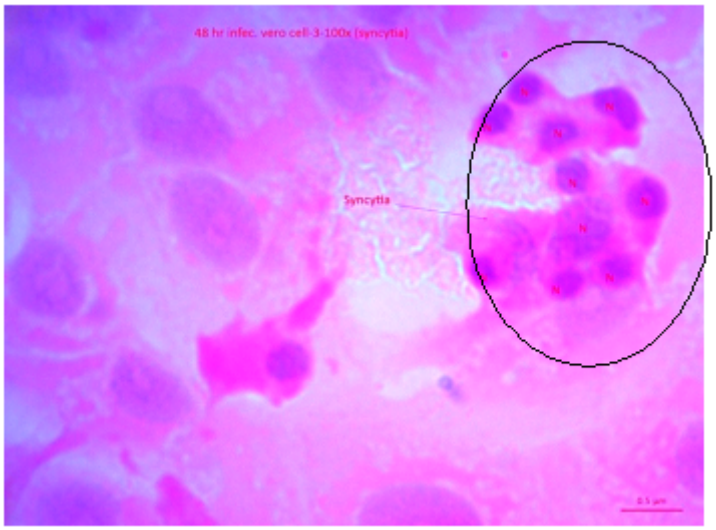

Figure 13

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

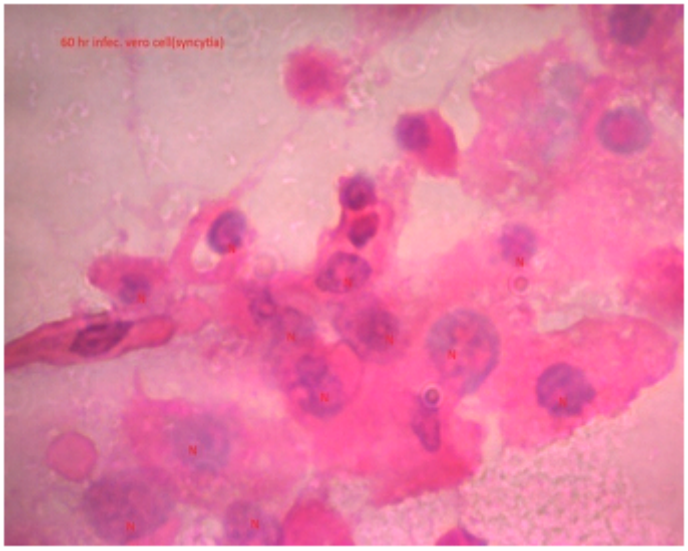

Figure 14

PPRV infected monolayer: large syncytia formation on 60 hpi at 60th passage level. H & E, x40

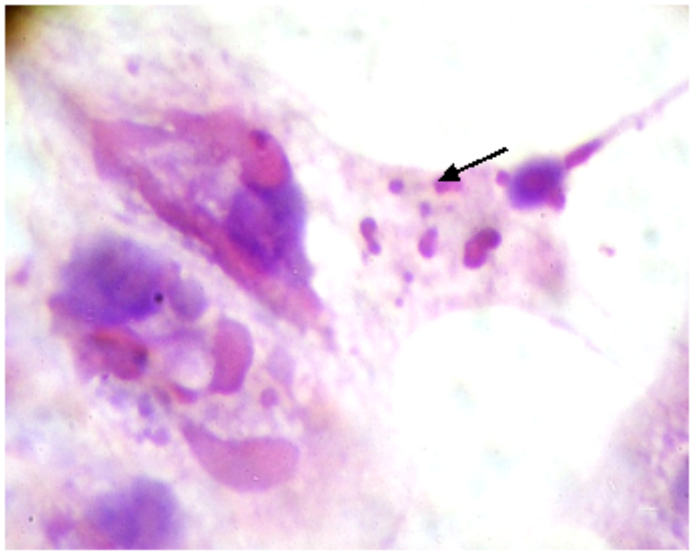

Figure 15

PPRV infected monolayer: several intracytoplasmic inclusion bodies on 72 hpi at 60th passage level. H &E, x100

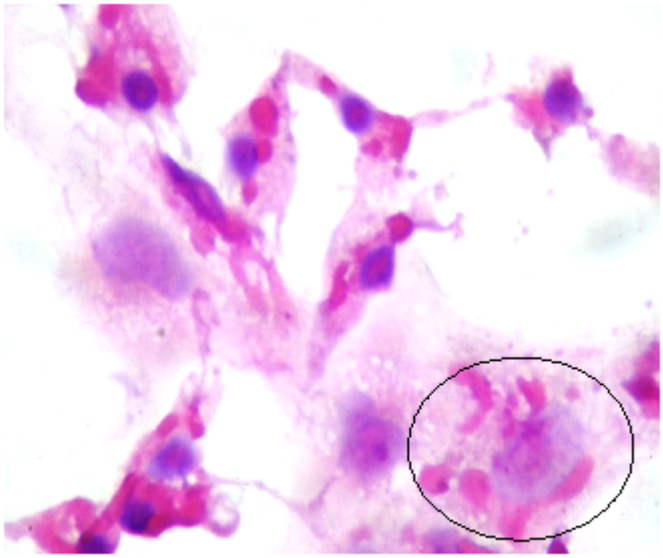

Figure 16

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

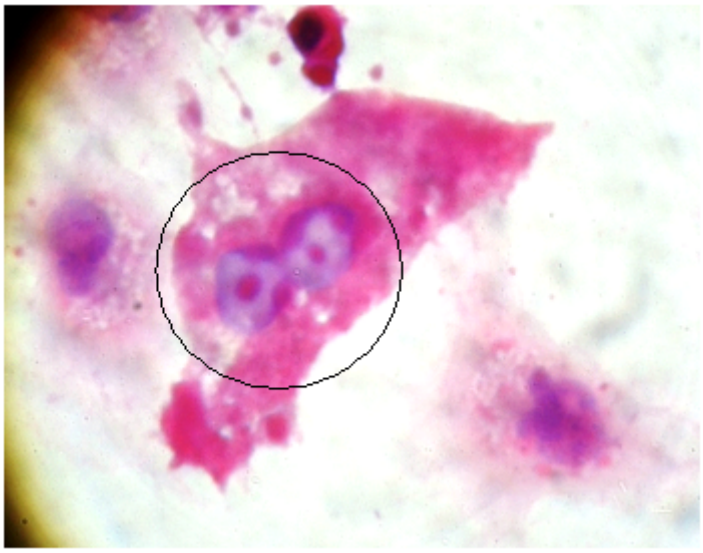

Figure 17

PPRV infected monolayer: deeply stained round large eosinophilic IB (circled area) on 72 hpi at 60th passage level. H & E, x 100

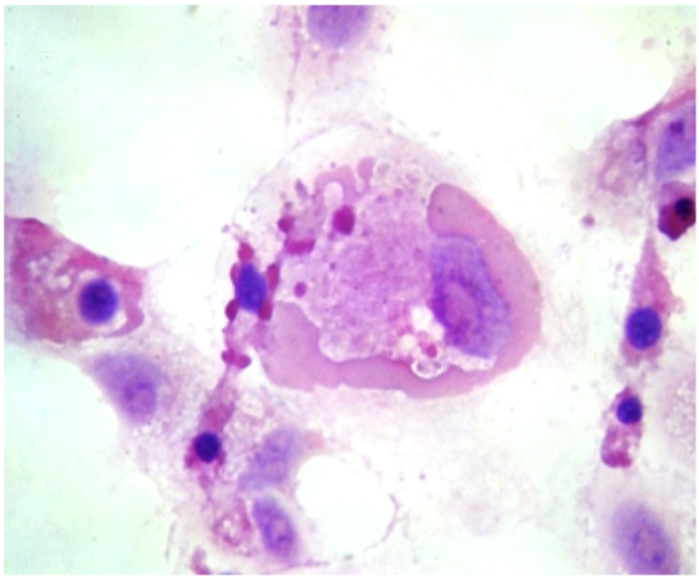

Figure 18

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

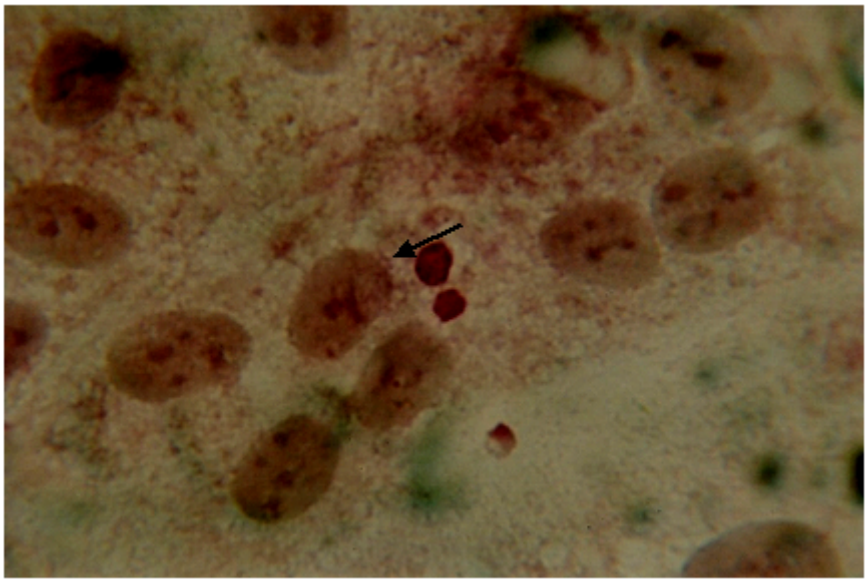

Figure 19

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