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Research Article

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Plaque Purification of Bluetongue Virus -12 (BTV-12)

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ABSTRACT

In the present study plaque purification of BTV-12 isolated from bluetongue outbreaks in 2016 of Kurnool was done along with studying its infection kinetics in Vero cell line. BTV-12 isolate adapted to Vero cell line had a titre of $10^{6.116}$/ml. Plaque purification was carried for three times using agarose overlaying method in 6 well plate. The plaque purified virus revealed 10 segmented genome on agarose gel electrophoresis. Plaque purification was confirmed since RT-PCR amplified only a product of 750 bp with BTV-12 specific primers. The purified virus was not amplified by other primers specific for BTV-1, 2, 4, 9, 10, 16, 21, 23 and 24. Furthermore, cytopathic effect of plaque purified BTV12 in Vero cell line was studied by H&E staining of infected Vero cells at 24, 36, 48 and 72 hrs post infection for understanding the kinetics of infection. The thus plaque purified virus can be used either to study pathogenesis or to raise hyper immune serum which can be used in neutralization assays to determine the serotype of unknown BTV.

Key words: *Orbivirus, Plaque purification, BTV serotypes, Infection kinetic, Hyper immune serum.*

INTRODUCTION

Bluetongue (BT) is an infectious, non-contagious arthropod borne viral disease of wild and domestic ruminants caused by bluetongue virus (BTV) the type species of the genus Orbivirus and belongs to the family Reoviridae[7]. BTV infects most of the domestic and wild ruminant and causes BT disease primarily in sheep characterised by severe clinical signs such as fever, lameness (coronitis), swelling and cyanosis of lips and tongue. It is listed as 'notifiable by office international des Epizooties[8]. It consists of ten segments of double-stranded RNA (dsRNA) encoding 7 structural proteins (VP1 to VP7) and 4 non-structural proteins (NS1, NS2, NS3 and NS3a)[13].

Currently, a total of 29 serotypes of BTV are recognized worldwide[6]. In India, 23 serotypes have been recognized based on serology and/ or virus isolation[14].

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Recently, Krishnajyothi *et al*[5], isolated BTV-24 from an outbreak during 2010 in Telangana State and Hemadri *et al*[3] isolated BTV-5 from Karnataka state. In endemic areas such as the southern parts of India, where mixed infections with more than one serotype have been commonly observed, it is mandatory to plaque purify the samples and typing of plaque purification virus by virus neutralization test (VNT) shall yield in accurate information regarding serotype prevalence[12]. In addition, the plaque purified virus can also be used in generating hyper immune serum specific to that serotype which can further be used in neutralization assays for typing of viruses. It is with these objectives that the current study was conducted.

MATERIAL AND METHODS

ADAPTATION OF BTV-12 TO VERO CELL LINE

BHK-21 cell line adapted BTV-12 serotype was passaged 3 times in Vero cells using Minimum Essential Medium (MEM) with 1% Foetal Bovine Serum (FBS). After completion of third passage, BTV-12 serotype tissue culture infective dose (TCID50) was calculated in 96 well tissue culture plate and according to Reed and Munch method[10], results were calculated.

PLAQUE PURIFICATION OF BTV

6-well tissue culture plates were used for plaque purification in which each well was seeded with $1X10^6$ Vero cells in 10% growth medium and incubated at 5% CO2 level at 37ºC. BTV-12 was serially diluted 10^{-1} from to 10^{-6} in plain medium, in six wells, monolayer of five wells were infected with 1ml of different virus dilution starting from 10^{-2} to 10^{-6} and 6th well was kept as cell control. This plate was kept in incubator with 5% CO2 at 37ºC for 1hr. involving swaying for every 10-15 minutes to ensure virus adsorption. After incubation, inoculum was drained off completely with the help of 1ml micropipette to remove unadsorbed virus without disturbing the monolayer and immediately overlayed with 10ml of 2X MEM with 2% FBS; 3% sea plaque agarose (Cambrex Bio Science, Cat.No.50100) mixture in 1:1 ratio. This draining off and overlaying was carried out for one well for each time instead of discarding all wells at a time to counter drying of monolayer as overlaying was done slowly. Finally, overlayed plates were allowed to solidify and then transferred to incubator. Plaques which are more distinct and isolated from others were collected with the help of micropipette in 200 µl of 10% MEM. Collected individual plaques were infected to Vero cells in 12-well plate for virus propagation.

MOLECULAR CONFIRMATION

RNA extraction

After third round of plaque purification, randomly one plaque was infected to T50 flask. Bluetongue virus genome i.e., double strand RNA (ds RNA) was extracted from cell culture fluid by Acid Phenol method using TRIZOL reagent and after confirmation the sample polymerase chain reaction (PCR).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complementary DNA synthesis (c DNA)

For cDNA synthesis a total of 30µl reaction mixture was prepared consisting 10µl of RNA, 9µl of RT mix and 11µl of nuclease free water.

This RNA mix was denatured at 95℃ for 5 minutes followed by snap cooling for 5 minutes. Meanwhile RT mix was prepared and stored at 4℃ until RNA denaturation. After snap cooling, RT mix was added to denatured RNA mix and subjected to following conditions in PCR thermocycler.

Annealing : 25℃/10 minutes
RT enzyme activation : 42℃/1 hour
RT enzyme inactivation : 72℃/10 minutes
Hold at 4℃

PCR SET UP:

Polymerase chain reaction(PCR) for plaque purified virus was carried out with primers (IDT-DNA) specific for available bluetongue virus serotypes (BTV-1, 2, 4, 9, 10, 12, 16, 21, 23, 24) and with positive and negative controls for each serotype.

PCR conditions followed in thermo cycler are:

For BTV-1,2,4,9,10,12,21,23,24		For BTV-16:	
94^0C for 3 minutes		94^0C for 3 minutes	
94^0C for 30 second	35 cycles	94^0C for 30 seconds	35 cycles
55^0C for 30 seconds		50^0C for 30 seconds	
72^0C for 1 min 30 sec		72^0C for 1 min 30 sec	
72^0C for 10 minutes		72^0C for 10 minutes	

PCR thermo cycler was set to hold at 4^oC after completion of reaction.

GROWTH CHARACTERS OF BTV -12 IN CELL LINES

Infected monolayers on coverslips were taken out carefully from 6 wells plate. The coverslips were washed thrice with PBS. Then the coverslips were fixed in methanol for five minutes. Later haematoxylin was added to the coverslips and incubated for 10 min. then the coverslips were immersed in water for 25 min. Later, eosin was added to the coverslips and incubated for 30 sec. Then the coverslips were washed with absolute alcohol for10 sec. After complete drying the coverslips were examined under the inverted microscope.

RESULTS

VERO CELL LINE INFECTION

BHK-21 adapted BTV-12 was adapted to Vero cells by passaging 3 times. Infection was done on 90% confluent monolayer of Vero cells. In the first passage, cytopathic effect was seen by 4th day of infection and complete CPE was observed by 5-6 days. In the next two passages, CPE was observed between 36-48 hrs.as rounding and clumping of dead cells. Complete peeling off of monolayer was observed within 72 hrs. of infection (Fig 1.A). Control monolayer didn‘t show any CPE (Fig 1.B).

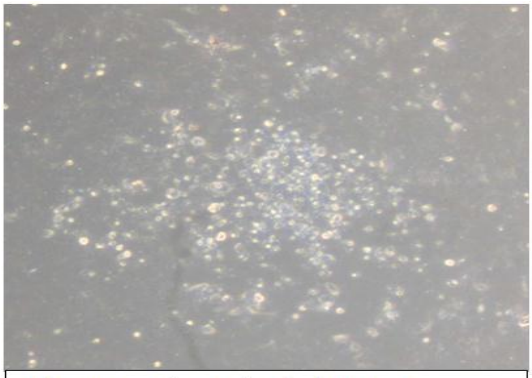

Fig 2 A- Single individual plaque surrounded with confluent healthy monolayer (40X)

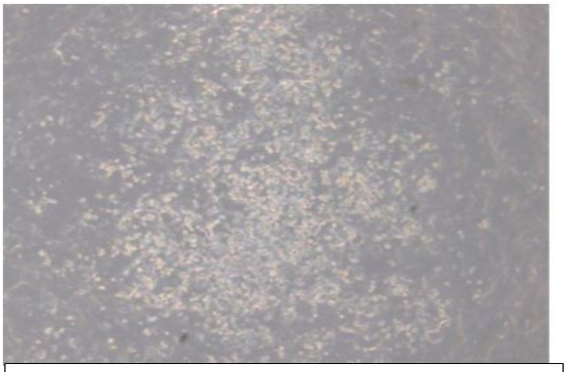

Fig 2B- Spreading plaque (40X)

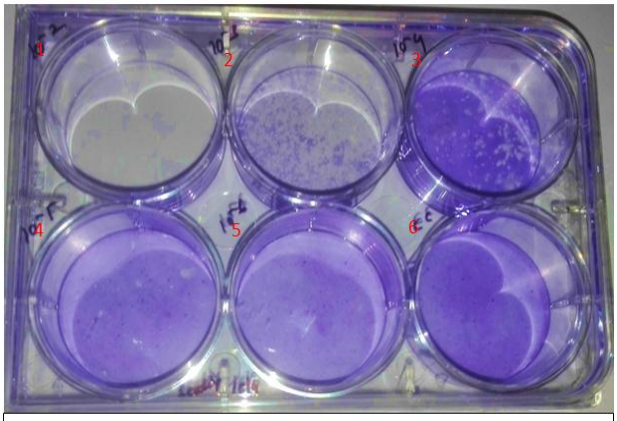

Fig 2C: Crystal violet staining of monolayer showing plaques
In above stained plate, wells numberd 1 to 5 infected with 10^{-2} to 10^{-6} dilutions of virus respectively and well no. 6 kept as control i.e., infected with plain MEM.

MOLECULAR CONFIRMATION

Agarose gel electrophoresis of RNA isolated from BTV infected Vero cells revealed segmented genome pattern with 8 clear and 2 indistinct bands (Fig 3) which was then used in RT-PCR as sample. For serotype confirmation, RT- PCR products were analysed by gel electrophoresis, only BTV-12 serotype specific primers showed specific PCR amplicon of 750bp with sample cDNA (Fig.4 A), and did not give any amplification with remaining serotype specific primers (Fig 4.B). Thus the isolate was confirmed as BTV-12.

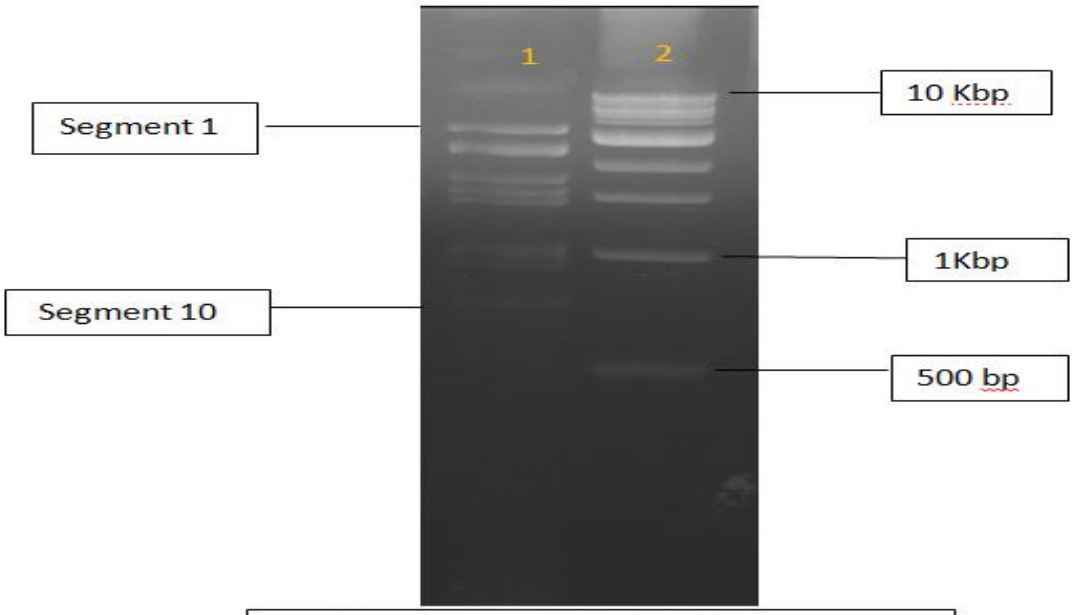

Fig 3: Agarose gel electrophoresis of plaque purified BTV-12 RNA
Lane 1- Segments of BTV 12 genome
Lane 2- 1 kbp ladder

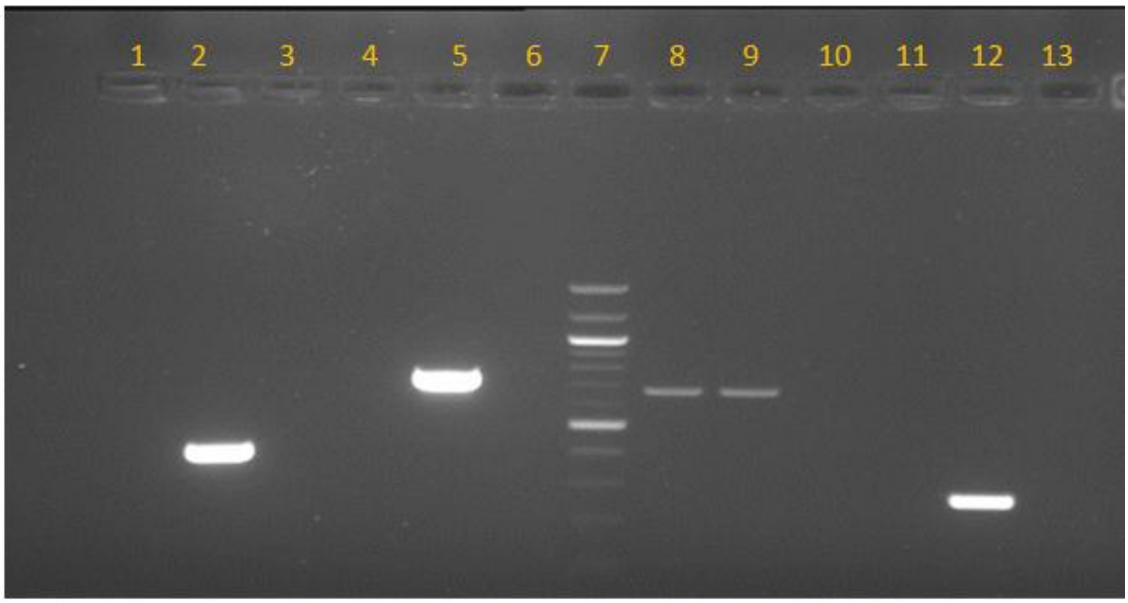

Fig 4 A: Gel electrophoresis of BTV-4, 10, 12 and 24.

Lane 1: Sample cDNA with BTV-4 primers	**Lane 2**: Positive control for BTV-4: 464 bp	**Lane 3**: Negative control
Lane 4: Sample cDNA with BTV-10 primers	**Lane 5**: Positive control for BTV-10: 800 bp	**Lane 6:** Negative control
Lane 7: 100 bp ladder		
Lane 8: Sample cDNA with BTV-12 primers	**Lane 9**: Positive control for BTV-12: 750 bp	**Lane 10**: Negative control
Lane 11:Sample cDNA with BTV-24 primers	**Lane 12**: Positive control for BTV-24: 319 bp	**Lane 13**: Negative control

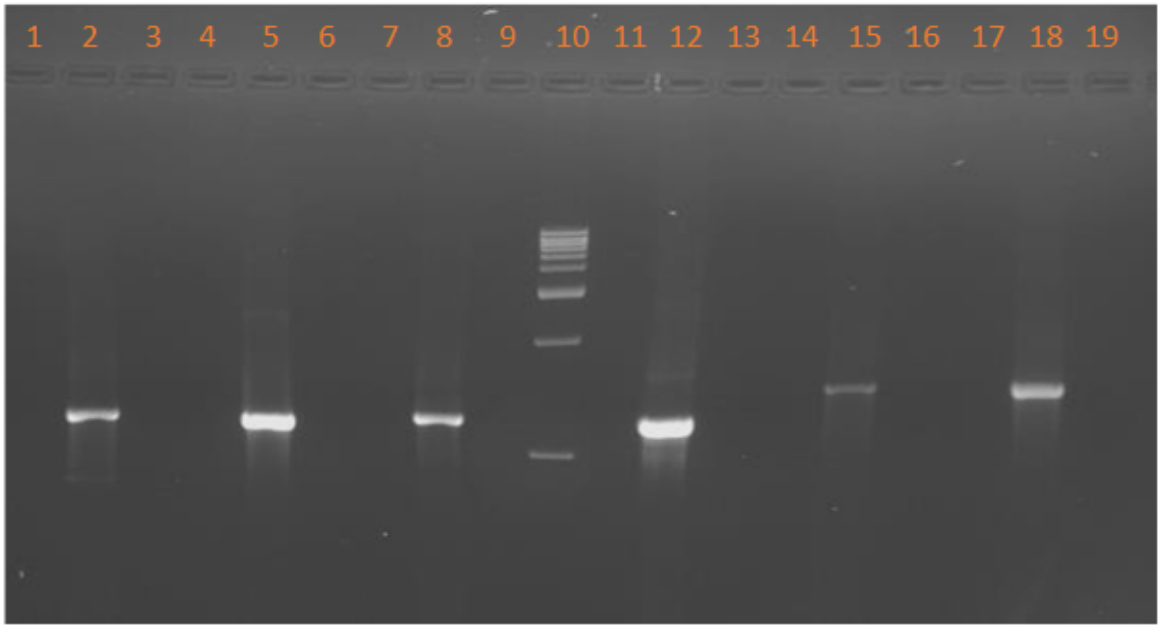

Fig. 4B: Gel electrophoresis of BTV-1, 2, 9, 16 21 and 23.

Lane 1: Sample cDNA with BTV-1 primers	**Lane 2**: Positive control for BTV-1: 1180 bp	**Lane 3**: Negative control
Lane 4: Sample cDNA with BTV-2 primers	**Lane 5**: Positive control for BTV-2: 1167 bp	**Lane 6**: Negative control
Lane 7: Sample cDNA with BTV-9 primers	**Lane 8**: Positive control for BTV-9: 1200 bp	**Lane 9**: Negative control
Lane 10: 1 kbp ladder		
Lane 11: Sample cDNA with BTV-16 primers	**Lane 12**: Positive control for BTV-16: 1200 bp	**Lane 13**: Negative control
Lane 14: Sample cDNA with BTV-21 primers	**Lane 15**: Positive control for BTV-21: 1388 bp	**Lane 16**: Negative control
Lane 17: Sample cDNA with BTV-23 primers	**Lane 18**: Positive control for BTV-23: 1370 bp	**Lane 19**: Negative control

GROWTH KINETICS OF BTV12 IN VERO CELLS

Cytopathic effect of BTV12 in Vero cell was studied by H&E staining of infected Vero cells at 24, 36, 48 and 72 hrs post infection (PI) for understanding the kinetics of infection. During 24 hrs of initial infection, there was no observed CPE (Fig 5.A& B) but 48hrs later, characteristic CPE with swollen spindle shaped cells which aggregated together into small and large clumps were observed (Fig 5.C& D). By 72hrs of PI, compledetachof the monolayer took place (Fig 5.E &F).

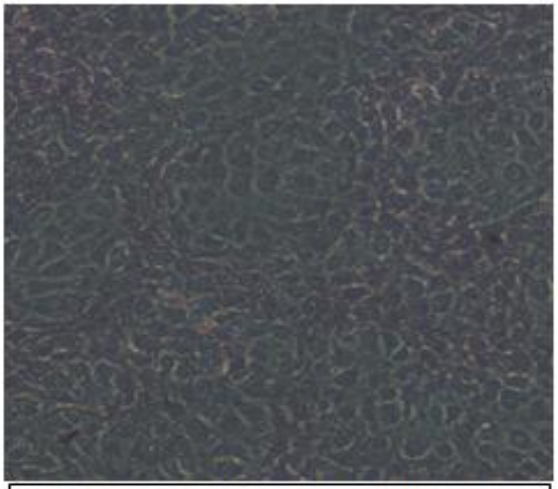

Fig 5A: Monolayer after 24hrs PI (100X)

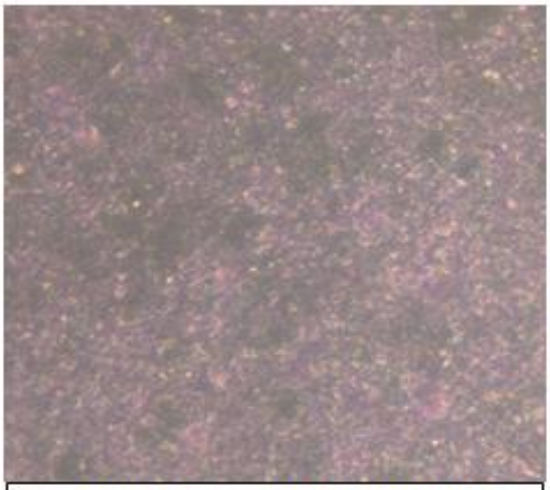

Fig 5B: Monolayer after 24hrs PI (40X)

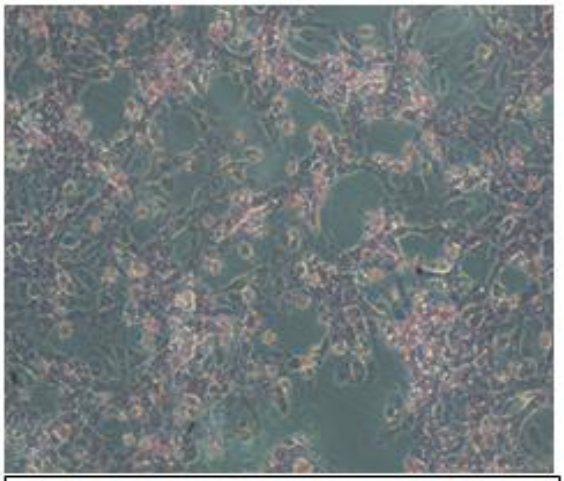

Fig 5C: Rounding and clumping of infected monolayer after 48 hrs. PI (100X).

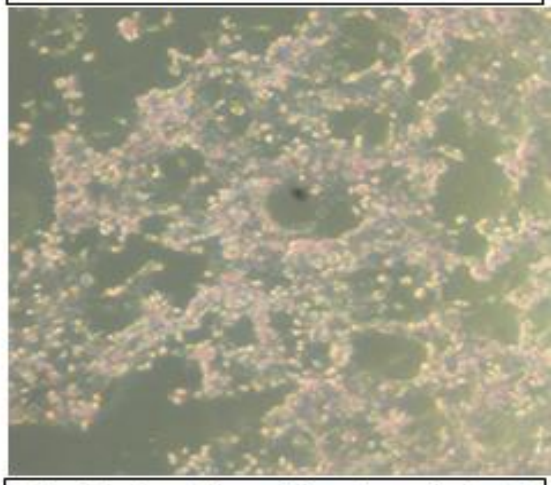

Fig 5 D: Rounding and clumping of infected monolayer after 48 hrs. PI (40X).

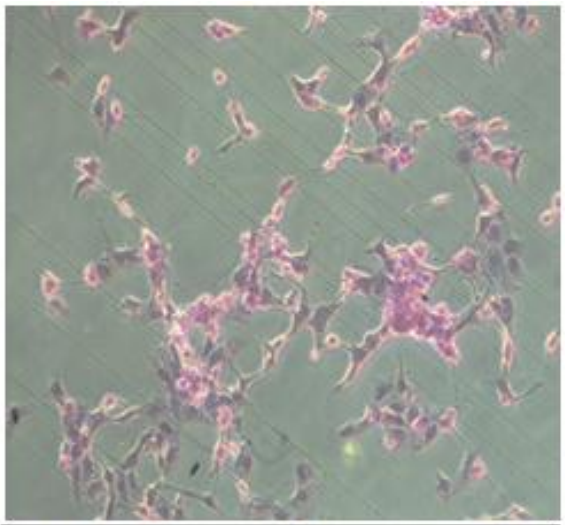

Fig 5 E: Complete detachment of infected monolayer after 72 hrs. PI (100X).

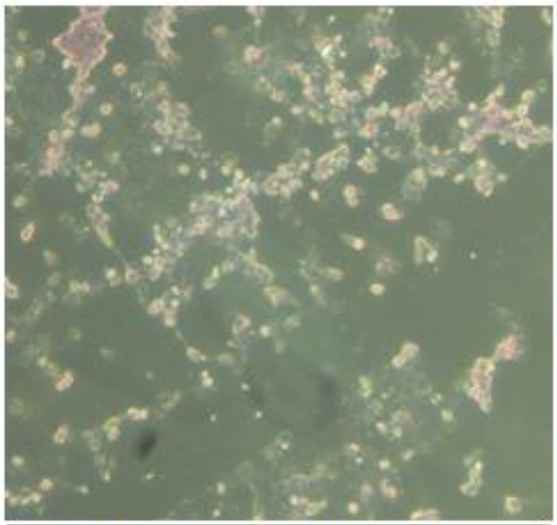

Fig 5 F: Complete detachment of infected monolayer after 72 hrs. PI (40X).

DISCUSSION

In the present study, agarose overlay method was used to plaque purify the BTV-12 in Vero cells for three times. Serial 10-fold dilutions of stock virus was made and 10^{-2} to 10^{-6} dilutions were infected on to monolayer of each well of 6-well plate. After 3 days of incubation suspected plaque areas of minute size were noticed which became more prominent by 4th day, a finding which can be correlated with the observations of Howell *et al*[4], Dulbecco[2] and Reddy[11].

Regarding the morphology, plaques observed in this study were almost clear, circular in shape with similar sizes (1-1.5 mm). However, increase in size was observed on prolonged incubation. The small size of plaques might be due to increased agarose concentration (1.2%) in overlay as observed by Howell *et al*[4] and Reddy[11].

For serotype confirmation, RT-PCR product was observed only with BTV-12 specific primers (750bp) by gel electrophoresis. No amplification was observed with primers specific for other serotypes. Amplification of positive cDNA of each serotype yielded expected size of products with respective primers. These results are in accordance with the conclusions of Prasad *et al*[9], Reddy *et al*[12] and Krishnajyothi *et al*[5], regarding VP2 based serotype-specific RT-PCR.

During 24 hrs of initial infection there is no observed CPE but 48hrs later characteristic CPE with swollen spindle shaped cells which were aggregated together forming small and large clumps was observed. By 72 hrs of PI, complete detachment of the monolayer took place. These results are in agreement with the observations made by Balam[1] and Subhadra[15]. They observed CPE with swollen spindle shaped cells and complete detachment of monolayer took place by 72 hrs PI.

CONCLUSION

BTV-12 was successfully plaque purified in Vero cells which can be used further in serological or pathogenesis studies.

Acknowledgments

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