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Plaque Purification of Bluetongue Virus Serotype-4 (BTV-4)

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A B S T R A C T

The present study was taken up with a view to undertake plaque purification of BTV-4 isolate available in the department, to confirm its purity. BTV-4 isolate was adapted to Vero cell line and had a titer of $10^{6.46}$/ml after 8 passages in the process of plaque purification which was determined as $10^{4.833}$/ml prior to adaption. Plaque purification was carried for three times using agarose overlaying method in 6 well plate. Each time plaques were observed from 4th day of infection of approximately not more than 1mm diameter. One of the plaques from third round of purification was grown in T_{25} flask and the resultant cell culture fluid was used for RNA isolation and RT-PCR through which the respective plaque was confirmed as BTV-4. Therefore, it was confirmed that there was no other serotype other than BTV-4 and the plaque purified virus can be used either to study pathogenesis or to raise hyperimmune serum.

Introduction

Bluetongue (BT) an infectious, non-contagious viral disease of sheep and other domestic and wild ruminants caused by bluetongue virus (BTV), is a segmented double-stranded RNA virus of genus *Orbivirus* under family *Reoviridae*. Segmented genome facilitates genetic reassortment between different serotypes and strains. At present 27 distinct BTV serotypes have been recognized worldwide with the possible addition of two more serotypes (Hofman *et al.,* 2008; Maan *et al.,* 2011; Zientara *et al.,* 2014). In India, 23 serotypes have been recognized based on serology, among them 14 serotypes viz., BTV-1, 2, 3, 4, 6, 9, 10, 12 16, 17,18, 21,23 and 24 have been isolated (AINP-BT, 2012; Rao *et al.,* 2014; Krishnajyothi *et al.,* 2016).

Due to a large number of susceptible hosts, BTV serotypes and *Culicoides* vectors; control of BT is a challenge. Therefore, effective control of BT disease requires proper diagnosis (Reddy *et al.,* 2010). A preliminary diagnosis of BT disease is usually done by epidemiology, clinical sign and post-mortem finding (Afshar, 1994). Proper diagnosis involves isolation of virus then standard

serological and molecular confirmation. But, due to limitations of serological and molecular techniques, a combination of Reverse transcription polymerase chain reaction (RT-PCR) with Virus neutralization test (VNT) may provide better results for which plaque purification of the samples is necessary (Reddy *et al.,* 2015). Hence, the current study was undertaken with an aim to plaque purify BTV4 isolated from BT outbreaks in Andhrapradesh during 2015.

Materials and Methods

Adaptation of BTV-4 to Vero cell line

BHK-21 cell line adapted BTV-4 serotype was passaged four times in Vero cells using Minimum Essential Medium (MEM) with 1% Fetal Bovine Serum (FBS). After fourth passage, BTV-4 serotype strength was determined in terms of tissue culture infective dose 50 (TCID50) in 96 well tissue culture plate.

After molecular confirmation, virus titer was calculated once again to know whether there was any increase in TCID50 as it was passaged 7 times more after Vero Cell line adaptation during plaque purification. Virus titer was calculated according to Reed and Munch method (1938).

Plaque purification of BTV

Plaque purification was carried out in 6-well tissue culture plates using agarose overlaying method. Each well was seeded with 3ml of growth medium (MEM with 10% FBS) containing Vero Cells ($4X10^6$ Vero cells per plate) and kept in incubator with 5% CO_2 at 37ºC. After attaining 80-90% confluence, BTV-4 was serially diluted from 10^{-1} to 10^{-6} in plain medium. After discarding growth medium from six well plate, monolayer of five wells were infected with 1ml of different virus dilutions starting from 10^{-2} to 10^{-6} whereas 6^{th} well was kept as cell control and kept in incubator with 5% CO_2 at 37ºC for 1hr. involving swaying for every 10-15 minutes to ensure virus adsorption. After 1hr. incubation, inoculum was drained off completely without disturbing the monolayer and each well was immediately overlaid with 3 ml of 2X MEM with 10% FBS; 2.6% 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. Thus, overlaid plates were allowed to solidification and then transferred to incubator. Plaques with more discretion from others were collected with the help of micropipette in 200 µl of growth medium with 10% FBS. Collected plaques were infected to Vero cell monolayers in 12-well plate to check whether those of virus origin or dead cells. Infected monolayers of 6-well tissue culture plate were stained with 1% crystal violet to observe plaques with unaided eye.

Molecular investigation

Genome extraction

Randomly one plaque was selected from third round of plaque purification and infected to T_{25} flask. BTV genome i.e., double strand RNA (ds RNA) was extracted from cell culture fluid by Acid Phenol method using Trizol^R reagent and after confirmation the same was used for polymerase chain reaction.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Complementary DNA (cDNA) was synthesized using a total of 30µl reaction mixture (10µl of RNA, 9 µl of RT mix; 11µl of nuclease free water).

First, RNA mix was denaturated at 95^{o}C for 5 minutes followed by snap cooling for 5 minutes. Meanwhile RT mix was prepared and stored at 4^{o}C until RNA denaturation. After snap cooling, RT mix was added to denatured RNA mix and subjected to following conditions in PCR thermo cycler with the following conditions: 25^{0}C/10 minutes, 42^{o}C/1 hour, 72^{o}C/10 minutes, Hold at 4^{o}C.

PCR

Polymerase chain reaction 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) (Reddy *et al.*, 2015) along with positive and negative controls for each serotype.

Results and Discussion

Adaptation of virus

During adaptation, in the first two passages, cytopathic effect (CPE) as rounding, clumping of dead cells and complete peeling off monolayer (Fig. 1) was observed 4-5 days post infection (dpi.) whereas in the following two passages time taken for complete CPE was only 3 dpi. Virus titer in terms of TCID50 was calculated as $10^{3.833}$/100 µl (or) $10^{4.833}$/ml.

Plaque purification

Plaques of approximately not more than 1mm in diameter were first identified after 3 dpi. (Fig. 2A) which became more prominent from 4th day onwards as restricted CPE areas showing dead cells scattering light surrounded by healthy monolayer (Fig. 2B). Plaques collected from 6-well plate infected to 12-well plate also showed CPE within 3 dpi., therefore confirming that collected plaques contained infectious virus.

Number of plaques was observed to decrease as dilution of virus increased which could be observed best at a glance after staining with 1% crystal violet solution (Fig. 3).

Among wells infected with 10^{-2} to 10^{-6} virus dilutions, only 10^{-2} to 10^{-4} dilutions produced plaques whereas monolayer infected with 10^{-5} and 10^{-6} dilutions revealed no plaques similar to uninfected well.

Molecular confirmation

Gel electrophoresis of RNA extracted from plaque purified BTV-4 infected cell culture fluid revealed clear segmented nature of genome (Fig. 4) which was then used in RT-PCR confirmation.

Gel electrophoresis of PCR products showed specific PCR amplicon of 464bp with BTV-4 serotype specific primers but did not give any amplification with remaining serotype specific primers (Fig. 5 and 6; Table 1). Thus, the isolate was confirmed as BTV-4 serotype. Finally, TCID50 of the RT-PCR confirmed virus was calculated as $10^{5.46}$/100 µl (or) $10^{6.46}$/ml.

PCR conditions followed in thermo cycler

For BTV-1,2,4,9,10,12,21,23,24

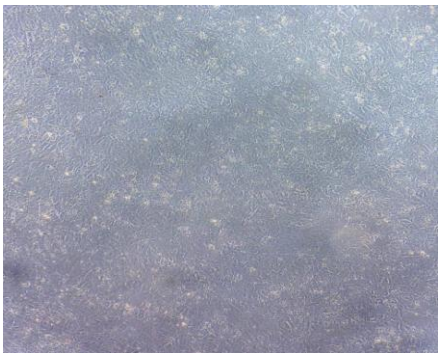
94^{0}C for 3 minutes
94^{0}C for 30 second
55^{0}C for 30 seconds
68^{0}C for 2 minutes
68^{0}C for 10 minutes
} 35 cycles

For BTV-16:

94^{0}C for 3 minutes
94^{0}C for 30 seconds
50^{0}C for 30 seconds
68^{0}C for 2 minutes
68^{0}C for 10 minutes
} 35 cycles

PCR thermo cycler was set to hold at 4^{o}C after completion of reaction.

1.A



1.B

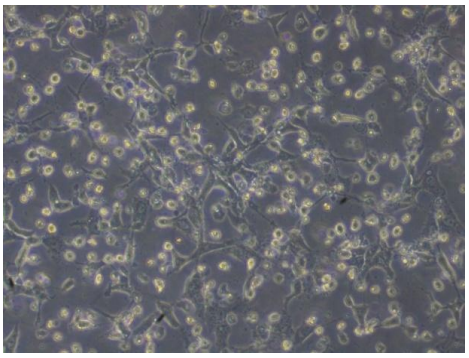
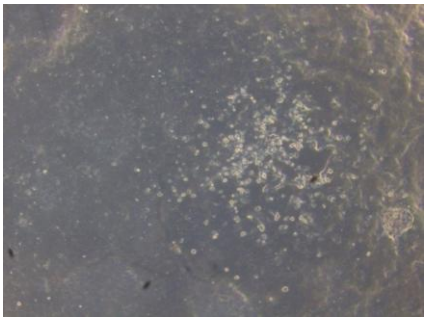


Figure 1: Propagation of BTV4 in Vero Cell line

1.A shows mock infected cell line exhibiting no CPE, whereas 1.B shows BTV 4 infected cell line on 5th day post infection showing characteristic CPE (40X).

2.A



2.B

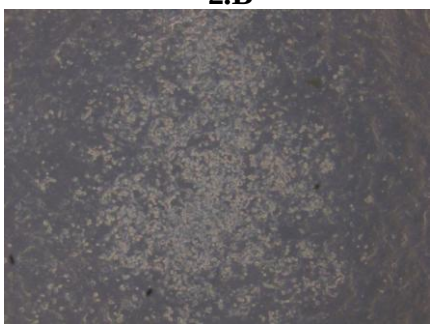


Fig 2: Plaques of BTV 4 as seen under inverted microscope (40X).
Fig 2A shows single individual plaque surrounded with confluent healthy monolayer.
Fig 2B shows spreading plaque

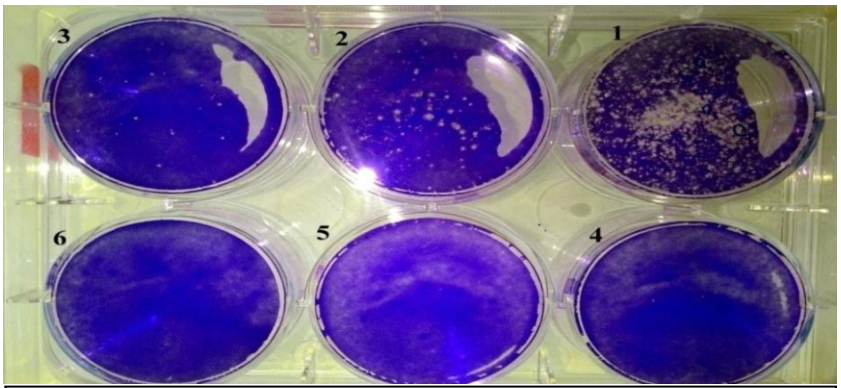


Fig 3: Crystal violet staining of monolayer showing plaques.
In above stained plate, wells numbered 1 to 5 were infected with 10^{-2} to 10^{-6} dilutions of virus, respectively and well no. 6 was kept as control i.e., infected with plain MEM. Plaques can be seen as white areas.

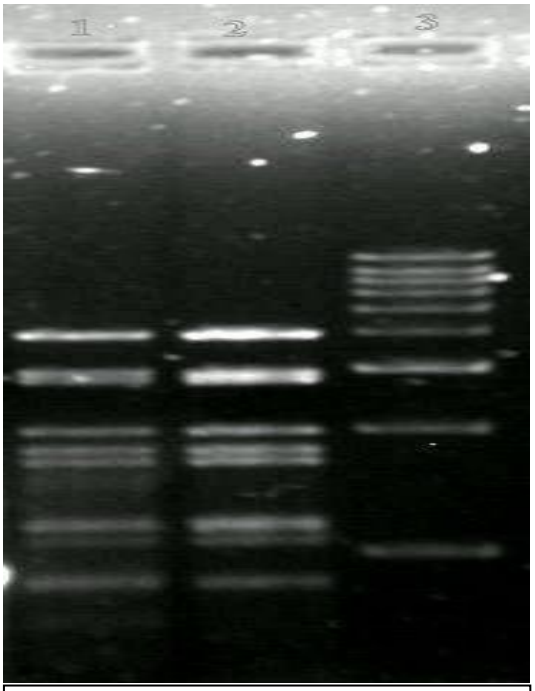


Fig. 4: Ten (10) segmented ds RNA pattern of BTV 4 genome.
Lane 1 &2 shows ten segmented pattern of BTV4 that was purified from two different plaques.
Lane 3 is 100bp DNA ladder.

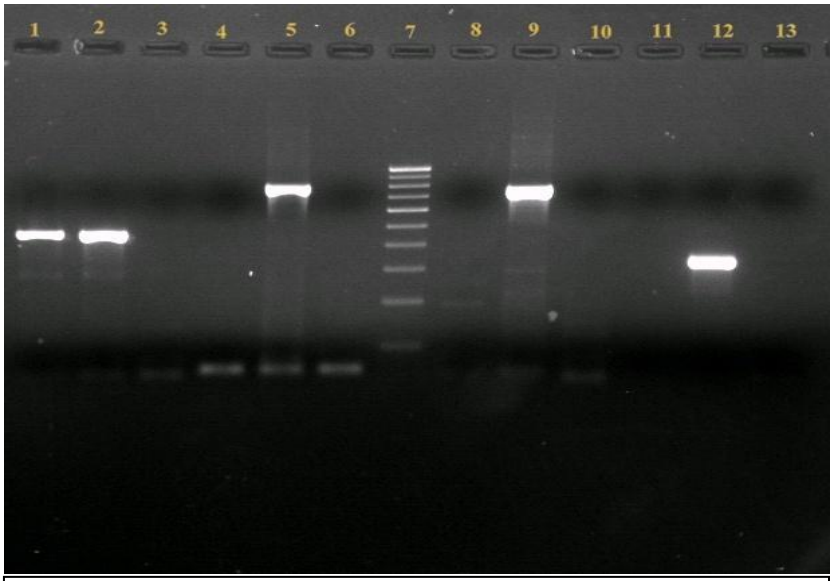


Fig 5: Molecular confirmation of BTV 4
Plaque purified BTV 4 was confirmed as BTV 4 when PCR amplicon was observed with BTV 4 specific primers but not with BTV 10,12 and 24 specific primers.

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

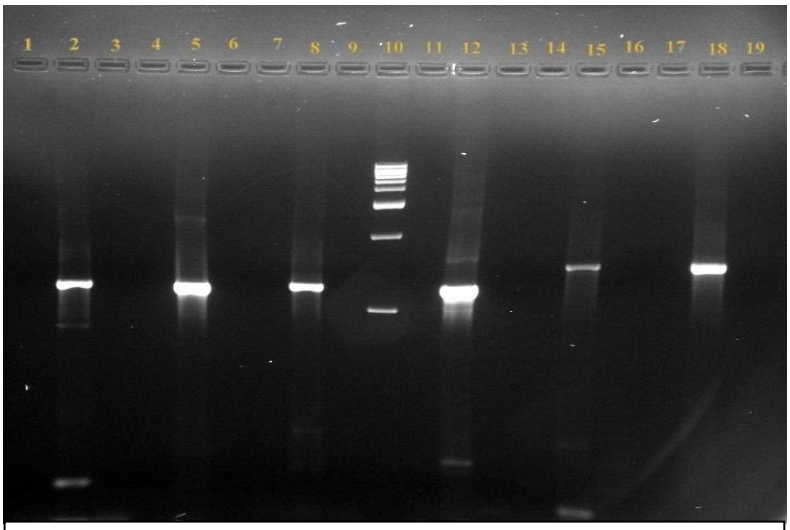


Fig 6: Molecular confirmation of BTV-4
Plaque purified BTV 4 was confirmed as BTV 4 when PCR amplicon was observed with BTV 4 specific primers but not with BTV 1,2, 9, 16, 21, and 23 specific primers.

Lane 1:Sample cDNA with BTV-1 primers	**Lane 2: Positive control for BTV-1: 1179 bp**	**Lane 3: Negative control**
Lane 4:Sample cDNA with BTV-2 primers	**Lane 5:** Positive control for BTV-2: 1172 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

Table.1 Primers sequence (5′ -3′) used for PCR

Bluetongue virus serotype	Primer	Sequence
BTV-1	F	5̓ TGT CGA GCC GAT TGA AGA TCC GTC 3̓.
	R	5̓ ATC GTC ATT CCG TCG TTG TGC G 3̓
BTV-2E	F	5̓ TAC GCA CCT CGT GAG AGA GA 3̓
	R	5̓ GTT GGA GGA ACC AAC TTC CA 3’
BTV-4	F	5̓ GTT GGA TCT GAG AAA TGG GT 3̓
	R	5̓ AAG ACA CGG ATA AGG ATT CG 3
BTV-9E	F	5̓ GAT GGA ACG GCT AAA CCA AA 3̓
	R	5̓ TGG ATA TTT GAC ACG AGC GA 3̓
BTV-10	F	5̓ TGT ATC GTT AAG GCG AGG TCA GCA 3̓
	R	5̓ TGT CTT CTA ACG GCC TCT CAC G 3̓
BTV-12	F	5̓ TTT AGG TGA CCA TGT GGA GAC G 3̓
	R	5̓ CAA CGC ACT TTC GCA AAA CC 3̓
BTV-16	F	5̓ TCG AGG AAA GCG GAT ACC ACG T 3̓
	R	5̓ CGT TGC GCT AAC TCG ACT TCG C 3̓
BTV-21	F	5̓ GCA GAT TCG TAC AAC CAA CGG CC 3’
	R	5̓ TTG GGA TTT GCG AGG CGC GA 3̓
BTV-23	F	5̓ GCG TTG CGA TGG ATG AGT TAG CA 3̓
	R	5̓ GGT GGT CAT CTC TTC ATC TTC GGG G 3̓
BTV-24	F	5̓ GTT TCA TGA ATT TGA AGG ACG 3̓
	R	5̓ ACC TTG TGA AAT CTT AGT YTT TG 3̓.

(Reddy *et al.*, 2015)

Identifying of plaques in the current study can be correlated with the observations of Howell *et al.*, (1967) and Dulbecco (1952). The incubation time for plaque development is higher when compared with Cooper's (1962) study in which plaques were produced within 40 hrs of incubation. This could be attributed to his modified basic method i.e., agar-cell suspension and different virus in a different cell line.

The small size of plaques might be due to increased agarose concentration (1.2%) in overlay as observed by Howell *et al.*, (1967) but clear demarcation of plaques from surrounding healthy monolayer was observed using 10% FBS. This observation was same as Blanchard and Stott (1989) studies. Further, almost parallel results were obtained with Howell (1967) and Dulbecco (1952) with regard to linear relationship between number of plaques and virus concentration inoculated to monolayer.

Amplification of partial VP2 in plaque purified BTV 4 with expected size of 464bp was observed only with BTV-4 specific primers and is in accordance with the conclusions of Rodriguez-Sanchez *et al.*, (2010) and Dadawala *et al.*, (2013) regarding VP2 based serotype-specific RT-PCR.

BTV-4 was successfully plaque purified in Vero cells which can be used further in serological or pathogenesis studies. Also, increase in virus titre was observed from $10^{3.833}$/100 µl (or) $10^{4.833}$/ml to $10^{5.46}$/100 µl (or) $10^{6.46}$/ml with serial passages.

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