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Studying the growth pattern of Bluetongue virus serotype 2 and 15 isolates in BHK_{21} cell lines

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Abstract
Bluetongue is an Office International Epizootics List A disease and described as an economically devastating affliction of sheep. The etiological agent of this disease is bluetongue virus which is the prototype species of the genus Orbivirus in the family Reoviridae. If the titre of the virus at various intervals is known, optimum inoculum and time of harvest can be calculated for maximum yield, which plays a crucial role in de If the titre of the virus at various intervals is known, optimum inoculum and time of harvest can be calculated for maximum yield development of vaccine. Therefore, an attempt was made to know the titre of the virus at periodic intervals of post infection by studying the growth pattern of two bluetongue virus vaccine strains. The titration of the two isolates M11 (BTV 2) and N12 (BTV 15) in BHK_{21} cells was done for 5 passages. The time and titres were estimated as $10^{4.55}$, $10^{4.68}$, $10^{6.12}$, $10^{6.72}$ and $10^{7.22}$ $TCID_{50}$/ml for M11 isolate (BTV 2) and $10^{2.69}$, $10^{3.58}$, $10^{5.64}$, $10^{7.55}$ and $10^{7.56}$ $TCID_{50}$/ml for N12 isolate (BTV 15). Further one step growth curve experiments were conducted for the isolates in BHK_{21} cells. Both the viruses had maximum titres at 48 h. The inoculum size had no significant effect on the harvest in the dilutions tested.

Keywords: Intervals is known, calculated for maximum, maximum yield

Introduction
Bluetongue (BT) is a major Office International Des Epizooties listed disease of sheep and goats and is endemic disease in India, causing significant economic losses to the sheep industry. The disease is caused by bluetongue virus belonging to the genus Orbivirus of family Reoviridae (Sonali *et al*., 2017 and OIE, 2018) [19, 1]. The disease is transmitted by culicoides midges and is endemic where culicoides species can survive (Susmitha *et al*., 2012) [2]. The BTV genome is composed of ten linear segments of double-stranded RNA (dsRNA). The 10 segments code for seven structural (VP1-VP7) and four non structural proteins (NS1,2,3,3A) (Bommineni *et al*., 2008., Rao PP *et al* 2012., Maan NS *et al*., 2012.) [3, 4, 5]. Currently, 29 distinct serotypes of BTV are reported to be circulating geographically (Thota *et al*., 2021) [7]. In India with a total of 24 serotypes are reported and the majority of the outbreaks were reported from southern states in Peninsular India (Thota *et al*., 2021 and Reddy Y V *et al*., 2016 [7, 8].
The first incidence of BTV was recorded in the state of Maharashtra in the year 1963 (Sapre, 1964) [9]. Bluetongue serotypes 2, 9 and 15 have been isolated from sheep in Andhra Pradesh during various out breaks (Bommineni *et al*., 2008) [3]. Attempts have also made to prepare an inactivated vaccine incorporating these serotypes. It is very restinent to undertake growth curve studies on virus isolates to come to a conclusion regarding the optimum inoculum size and correct time of harvest for vaccine manufacturer**.** Keeping this in view growth curve studies were taken up on these isolates**.**

Materials and Methods
BTV serotype 2 M11 strain was isolated from sheep in the year 2003 during the outbreaks in Mahaboobnagar district of Andhra Pradesh (Yugender Reddy, 2004) [11]. BTV serotype 15 N12 isolated from sheep during bluetongue outbreaks in Nalgonda district of Andhra Pradesh in 2003 (Yugendar Reddy, 2004) [11].

BHK_{21} cell lines obtained from Veterinary Biologicals Research Institute (VBRI), Shantinagar, Hyderabad were used for propagation of BTV. Cell line was subcultured using MEM (Earle's MEM, GIBCO, Cat. No. 41500-034) supplemented with 8 percent foetal bovine serum (Biowest Cat No. S1810) and incubated at 37 °C.

Cultivation of BTV isolates in BHK_{21} cell lines: BTV 2 and 15 isolates (M11 and N12 isolates respectively) were serially passaged in BHK_{21} cell lines up to 5 passsages. The cell lines were inoculated with 0.1ml of virus and kept for adsorption for 1 hour. After adsorption 2% Minimum essential medium was added. The growth of the virus was confirmed by observing CPE in unstained and stained (H&E) monolayer preparations.

One step growth curve: Study was conducted in BHK_{21} monolayers in 96 well Tissue culture plates. To study the growth pattern of the BTV-2 and 15 (M11 and N12 isolates respectively), in BHK-21 cell lines, the cells were infected with BTV-2 (7.22 and 6.22 titre $\log_{10}$ $TCID_{50}$/ml) and BTV-15 7.56 and 6.56 titre $\log_{10}$ $TCID_{50}$/ml) separately. After allowing for adsorption of virus at 37^0C for 60 min, maintenance medium was added to each well. The samples of infected cultures were collected at every 12 h. up to 72 h. It was so planned that one plate would be harvested at one collection time. Supernatant of the wells was collected for cell free virus assay while the monolayers were freeze thawed thrice and collected for cell associated virus assay. These samples were titrated for virus in BHK_{21} cell lines. The $TCID_{50}$ was calculated by using Reed and Muench method (1938) [12].

Results and Discussion

Cultivation of BTV isolates in BHK_{21} cell lines: CPE at every 12 h. intervals were observed up to 72 h. PI after infecting the BHK_{21} cell line with BTV serotypes 2 and 15 (M11 and N12 isolates respectively). Distinct cytopathic effect appeared in the infected BHK_{21} cell monolayers with the BTV isolates from 36 h. PI. The characteristic changes observed were that of scattered focal rounding and aggregation of cells followed by cell destruction with foamy degeneration, loss of cell architecture accompanied by cell detachment. No changes were noticed in uninfected control monolayer. Similar pattern of CPE was also observed by Sreenivasulu (1995) [10], Deshmukh and Gujar (1999) [13] and Jain *et al.* (1986) [14]. The two BTV serotypes BTV-2 and BTV-15 (M11 and N12 respectively) were titrated in BHK_{21} cells. The infectivity titres of BTV serotypes are depicted in Table 1. Titres of BTV-2 ranged from $10^{4.55}$ in first passage to $10^{7.22}$ in fifth passage while titres of BTV-15 ranged from $10^{2.69}$ t0 $10^{7.56}$. There was a linear increase in the titre up to 5^{th} passage level and similar results were noticed by Roy and Mehrotra (1999) [15].

One step growth curve: The BHK_{21} cell lines infected with 7.2 $\log_{10}$ $TCID_{50}$/ml and 6.2 $\log_{10}$ $TCID_{50}$/ml of BTV-2 (M11) and 7.56 $\log_{10}$ $TCID_{50}$/ml and 6.56 $\log_{10}$ $TCID_{50}$/ml of BTV-2 (N12). The samples collected at various intervals of post-infection were titrated in BHK_{21} cell line.

The peak titres of BTV-2 (M11) CA as well as CF were noticed at 48 h PI. Further the titre of CA (7.35 $\log_{10}$ $TCID_{50}$/ml) virus was higher than that of CF (7.2 $\log_{10}$ $TCID_{50}$/ml) virus. Total yield (cell associated and cell free) of 7.58 $\log_{10}$ $TCID_{50}$/ml was observed at 48 h. PI in BHK_{21} cells infected with BTV-2 (M11) with titre of 7.2 $\log_{10}$ $TCID_{50}$/ml which declined to 6.06 $\log_{10}$ $TCID_{50}$/ml at 72 h. PI. (Table 2, Fig 1). Whereas 6.2 $\log_{10}$ $TCID_{50}$/ml inoculum size of BTV 2 (M11) was given, a total yield of 6.71 $\log_{10}$ $TCID_{50}$/ml at 48 h. PI. This later decreased to 4.8 $\log_{10}$ $TCID_{50}$/ml at 72 h. PI (Table 3, Fig 2).

The highest titre of BTV-15 (N12) CA as well as CF was noticed at 48 h. PI. The titre of CA (7.6 $\log_{10}$ $TCID_{50}$/ml) virus was higher than that of the CF (7.5 $\log_{10}$ $TCID_{50}$/ml) virus. Total yield (cell associated and cell free) of 7.85 $\log_{10}$ $TCID_{50}$/ml was observed at 48 h. PI in BHK_{21} cells infected with BTV-15 (N12) with titre of 7.56 $\log_{10}$ $TCID_{50}$/ml. This declined to 5.71 $\log_{10}$ $TCID_{50}$/ml at 72 h. PI. (Table 4, Fig 3).

Table 1: Titres of BTV 2 and 15 in BHK_{21} cell upto 5^{th} passage level

S.no	Serotype	Titre TCID50/ml at different passage levels				
		1st	2nd	3rd	4th	5^{th}
1	BTV-2 (M11)	$10^{4.55}$	$10^{4.68}$	$10^{6.12}$	$10^{6.72}$	$10^{7.22}$
2	BTV-15 (N12)	$10^{2.69}$	$10^{3.58}$	$10^{5.64}$	$10^{7.55}$	$10^{7.56}$

With BTV-15 (N12) at inoculum size of 6.56 $\log_{10}$ $TCID_{50}$/ml was inoculated in BHK_{21} cell line, Total yield of 6.85 $\log_{10}$ $TCID_{50}$/ml was observed at 48 h. PI. This later decreased to 4.91 $\log_{10}$ $TCID_{50}$/ml at 72 h. PI. (Table 5, Fig 4). Similar pattern of results were also noticed by Garg and Prasad (1996) [16] for bluetongue virus in mononuclear cells. Bhat *et al.* (1996) [17] noticed maximum titres at 48 h.PI in BHK_{21} cells and 60 h.PI in vero and C6/36 cells. Cromack *et al.* (1971) [18] while conducted one step growth curve studies on BTV noticed that from 5 to 15 h.PI, the titre of the released virus remained constant. However, after 15 h.PI there was a secondary 10 fold (one log) rise in the concentration of released virus. Further, he also observed that release of CA virus did not occur till 7 h.PI and the titre of the CA virus was maximal after 21 h.PI. However this may be because of different cell line (Madin-Darby bovine kidney cells) employed in their study.

Table 2: Result of one step growth curve of BTV-2 (M11) in BHK-21 cells (infected with $10^{7.2}$ $TCID_{50}$/ml)

Hours PI	12	24	36	48	60	72
CF virus ($TCID_{50}$ /ml)	$10^{4.5}$	$10^{5.5}$	$10^{6.36}$	$10^{7.2}$	$10^{6.5}$	$10^{5.24}$
CA virus ($TCID_{50}$ /ml)	$10^{4.76}$	$10^{5.75}$	$10^{6.63}$	$10^{7.35}$	$10^{6.88}$	10^{6}
Total virus ($TCID_{50}$/ml)	$10^{4.95}$	$10^{5.94}$	$10^{6.81}$	$10^{7.58}$	$10^{7.03}$	$10^{6.06}$

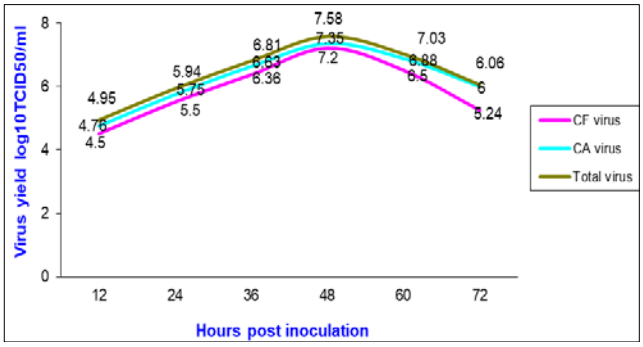

Fig 1: One step growth curve for BTV-2 (M11) virus in BHK-21 cells (infected with $10^{7.2}$$TCID_{50}$/ml)

Table 3: Result of one step growth curve of BTV-2 (M11) in BHK-21 cells (infected with $10^{6.2}$ $TCID_{50}$/ml)

Hours PI	12	24	36	48	60	72
CF virus ($TCID_{50}$ /ml)	$10^{3.6}$	$10^{4.22}$	$10^{5.32}$	$10^{6.3}$	$10^{5.5}$	$10^{4.52}$
CA virus ($TCID_{50}$ /ml)	$10^{3.87}$	$10^{4.35}$	$10^{5.5}$	$10^{6.5}$	$10^{5.7}$	$10^{4.63}$
Total virus ($TCID_{50}$/ml)	$10^{4.05}$	$10^{4.59}$	$10^{5.72}$	$10^{6.71}$	$10^{5.91}$	$10^{4.8}$

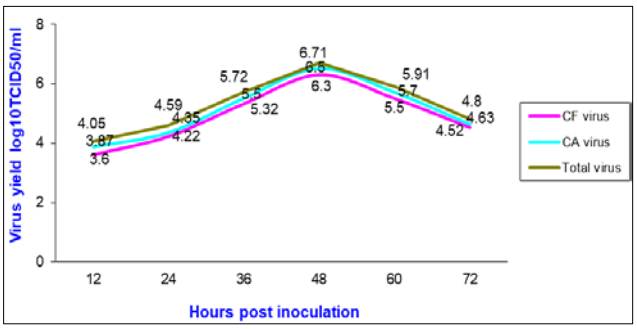

Fig 2: One step growth curve for BTV-2 (M11) virus in BHK-21 cells (infected with $10^{6.2}$$TCID_{50}$/ml)

Table 4: Result of one step growth curve of BTV-15 (N12) in BHK-21 cells (infected with $10^{7.56}$ $TCID_{50}$/ml)

Hours PI	12	24	36	48	60	72
CF virus ($TCID_{50}$ /ml)	$10^{4.6}$	$10^{5.3}$	$10^{6.5}$	$10^{7.5}$	$10^{6.67}$	$10^{5.3}$
CA virus ($TCID_{50}$ /ml)	$10^{4.78}$	$10^{5.8}$	$10^{6.7}$	$10^{7.6}$	$10^{6.8}$	$10^{5.5}$
Total virus ($TCID_{50}$/ml)	10^{5}	$10^{5.91}$	$10^{6.91}$	$10^{7.85}$	$10^{7.04}$	$10^{5.71}$

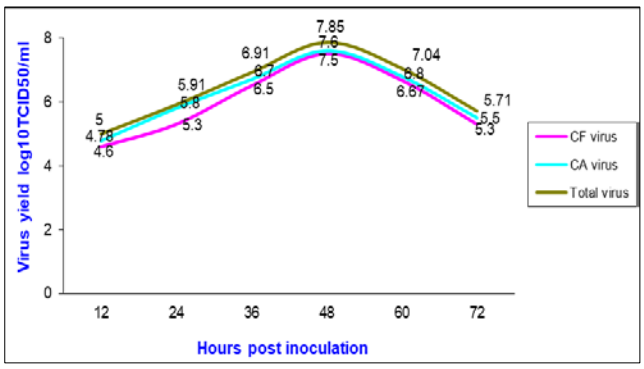

Fig 3: One step growth curve for BTV-15 (N12) virus in BHK-21 cells (infected with $10^{7.56}$$TCID_{50}$/ml)

Table 5: Result of one step growth curve of BTV-15 (N12) in BHK-21 cells (infected with $10^{6.56}$ $TCID_{50}$/ml)

Hours of PI	12	24	36	48	60	72
CF virus ($TCID_{50}$ /ml)	$10^{3.7}$	$10^{4.5}$	$10^{5.4}$	$10^{6.5}$	$10^{5.3}$	$10^{4.5}$
CA virus ($TCID_{50}$ /ml)	$10^{3.86}$	$10^{4.7}$	$10^{5.5}$	$10^{6.6}$	$10^{5.4}$	$10^{4.7}$
Total virus ($TCID_{50}$/ml)	$10^{4.08}$	$10^{4.91}$	$10^{5.75}$	$10^{6.85}$	$10^{5.65}$	$10^{4.91}$

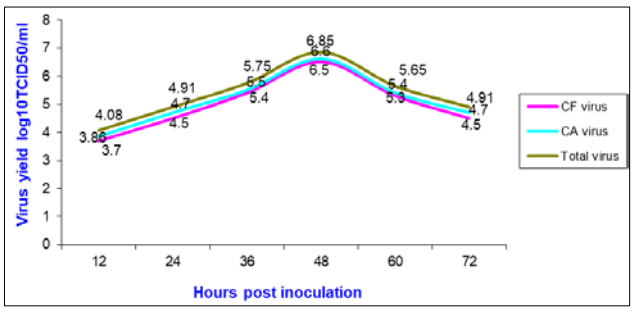

Fig 4: one step growth curve for BTV-15 (N12) virus in BHK-21 cells (infected with 106.56TCID50/ml)

Conclusion

In conclusion, two BTV vaccine strains M11 and N12 belonging to BTV-2 and BTV-15 respectively were characterized with respect to their titres at different passages, cytopathogenicity in BHK_{21} cells and single step growth curve of these vaccine strains also studied. The present available data is not sufficient to give firm conclusions. Further detailed studies are required in order to formulate suitable vaccine for control of BTV.

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