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Bagaza virus inhibits Japanese encephalitis & West Nile virus replication in *Culex tritaeniorhynchus* & *Cx. quinquefasciatus* mosquitoes

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***Background & objectives*: Studies have shown that certain flaviviruses influence susceptibility of mosquitoes by inhibiting/enhancing replication of important flaviviruses. Hence, a study was designed to determine whether Bagaza virus (BAGV), a flavivirus isolated from *Culex tritaeniorhynchus* mosquitoes in India, alters susceptibility of *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes to Japanese encephalitis (JEV) and West Nile viruses (WNV).**

***Methods*: JEV and WNV infection in *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes in the presence of BAGV was carried out by intrathoracic (IT) inoculation and oral feeding methods. Mosquitoes were infected with BAGV and WNV/JEV either simultaneously or in a phased manner, in which mosquitoes were infected with BAGV by IT inoculation followed by super-infection with JEV/WNV after eight days post-infection (PI). JEV and WNV yield on 7th and 14th day PI after super-infection was determined by 50 per cent tissue culture infective dose ($TCID_{50}$) method.**

***Results*: In *Cx. tritaeniorhynchus* mosquitoes, prior infection with BAGV significantly reduced JEV and WNV replication while in *Cx. quinquefasciatus*, BAGV influence was only seen with WNV. Reduction in virus titre was observed in IT inoculated and oral fed mosquitoes irrespective of the infection mode. JEV replication was also found reduced in *Cx. tritaeniorhynchus* mosquitoes persistently infected with BAGV at passage four.**

***Interpretation & conclusions*: BAGV infection in *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes altered their susceptibility to JEV and WNV producing low virus yield. However, the role of BAGV in inhibiting JEV/WNV replication in field mosquitoes needs further investigations.**

Key words Bagaza virus - intra thoracic inoculation - Japanese encephalitis virus - super infection - West Nile virus

Bagaza virus (BAGV), a mosquito borne arbovirus of genus *Flavivirus*, family *Flaviviridae* has been isolated repeatedly from different species of mosquitoes in Africa since its discovery from Bagaza district of Central African Republic in 1966[1]. Initial studies have shown that BAGV is antigenically close to Israel turkey meningoencephalitis virus, an important viral pathogen of poultry in Middle East and South

Africa[2]. The virus had a wide geographic distribution in West Africa as isolations were made from Central African Republic, Senegal, Cameroon and Mauritania involving more than seven mosquito species[3-5]. BAGV has never been associated with any outbreak of either human or veterinary importance despite its presence in the African continent for the last five decades until the detection of its role in the unusually high bird deaths (Partridges and pheasants) reported from Spain in 2010[6].

In India, BAGV was isolated from a pool of *Culex tritaeniorhynchus* mosquitoes collected during an outbreak of Japanese encephalitis virus (JEV) in Kerala[7]. Subsequent serological studies have demonstrated the presence of BAGV antibodies in 15 per cent of the human population in the area demonstrating subclinical infections in man[1,7]. *Culex tritaeniorhynchus* is the purported vector of JEV and is also a potential vector for West Nile virus (WNV). Some novel flaviviruses have been found to interact with pathogenic flaviviruses in mosquitoes. This study was, therefore, undertaken to determine the role of BAGV in replication of JEV and WNV in two important vector mosquitoes, *i.e. Cx. tritaeniorhynchus* and *Cx. quinquefasciatus*.

Material & Methods

The study was conducted at the Microbial Containment Complex, National Institute of Virology (NIV), Pashan, Pune, India. Infected mosquitoes were kept in plastic jars inside mosquito cages and all these experiments were carried out inside a bio-safety level-2 laboratory. Normal as well as infected mosquitoes were maintained at 28±2°C with 80±5 per cent relative humidity and 12:12 h light: dark cycle.

Viruses:

(i) BAGV - BAGV strain No. BAG96363, isolated from a pool of *Cx. tritaeniorhynchus* mosquitoes in 1996[1] was used in the study. This isolate has undergone six passages in Baby hamster kidney (BHK-21) cell line. The virus at P-7 (stock) had a titre of 5.0 $\log_{10}$ 50 per cent tissue culture infective dose ($TCID_{50}$)/ml.

(ii) JEV - Strain No. 057434, a human isolate obtained from Gorakhpur, India, in 2005 (NIV, unpublished data) has undergone several passages in mice and two passages in Vero E6 cell line. The stock virus prepared in Vero E6 cell line had a titre of 7 $\log_{10}$ $TCID_{50}$/ml.

(iii) WNV - The prototype strain of WNV (Eg101) procured from NIV virus repository was used in the study. The strain has undergone several passages in mouse brain and passaged once in Vero E6 cell line for stock preparation. The virus stock had a titre of 8.3 $\log_{10}$ $TCID_{50}$/ml.

Virus stock preparation: BHK-21 cell line was preferred for stock preparation and virus titration based on the high virus yield in the cell line (unpublished data). To prepare virus stock, BHK-21 cells grown in 225 cm^2 bottles were infected with 1MOI (multiplicity of infection) of BAGV as described earlier[1]. The cultures were observed daily for appearance of cytopathic effect (CPE) and when 70-80 per cent cells showed CPE, cultures were harvested. Virus was extracted from cells by freeze-thawing thrice and collecting the supernatant after centrifugation at 2790x *g* in a refrigerated (+4°C) centrifuge (Hettich, Germany) for 20 min. One ml aliquots of the suspension was made in sterile vials and stored at -86°C. One of the aliquots was titrated in the same cell line and virus titre was determined as mentioned earlier. BHK-21 cell line was maintained in minimum essential medium (MEM) supplemented with 10 per cent foetal bovine serum (FBS) and was passaged at every 3-4 days. Both MEM and FBS used for cell line maintenance were procured from Invitrogen, USA.

Insect flavivirus free mosquitoes were procured from the NIV insectary maintained at 28±2°C with 80±5 per cent relative humidity and 12:12 h light: dark cycle. Mosquito larvae were fed on a mixture of yeast powder and dog biscuit (3:1 w/w) while adults were maintained on a diet of 10 per cent glucose. Female mosquitoes were provided with 5-6 wk old chickens for blood meal on alternate days.

Infection of mosquitoes with virus: Infection of mosquitoes was carried out by intrathoracic inoculation (IT) and oral feeding. For IT inoculation, 50 mosquitoes for each experiment were immobilized by keeping them in ice for 5-10 min and inoculated intrathoracically with 100 plaque forming units (pfu) of BAGV inside a BSL-2 laboratory following the method described by Rosen and Gubler[8]. For oral feeding, mosquitoes (n=50 for each experiment) were starved for 12 h and allowed to feed on blood virus mixture through a chicken membrane as previously described[1]. Fully engorged mosquitoes were separated and used for the study. Infected mosquitoes (both IT inoculated and oral fed) were secured in plastic mosquito holding jars inside double walled mosquito cages and incubated at

28±2°C with 70-80 per cent humidity. Five mosquitoes were harvested at scheduled intervals and stored at -80°C. After completion of the experiment, mosquitoes of each day post-infection (PI) were triturated in 1 ml MEM containing 2 per cent FBS using a chilled mortar and pestle. The mosquito suspension was centrifuged; Millipore filtered (pore size=0.22 µm), diluted serially (ten-fold) and titrated in BHK-21 cells in quadruplicate. The cultures were observed daily, readings (cells with cytopathic effects) were scored, stained with amido black and virus titre of each day PI was determined[9]. All experiments were carried out in triplicate.

Determination of JEV/WNV replication in BAGV infected mosquitoes: The influence of BAGV in replication of JEV and WNV was studied using two methods. In the first method, mosquitoes were infected simultaneously with a mixture of BAGV and JEV or WNV by IT inoculation. In the second method, mosquitoes were infected with BAGV, incubated for eight days and superinfected with JEV/WNV. The infections were made by IT inoculation. In the oral infection method, mosquitoes were fed blood-virus mixture as described earlier. In all the cases, mosquitoes were incubated further, harvested on days 7 and 14 of infection with JEV/WNV, titrated and determined virus titre as described earlier. JEV/WNV yield in BAGV infected mosquitoes was compared with virus yield of mosquitoes infected with JEV/WNV alone and determined the influence of BAGV in replication of JEV/WNV.

Determination of effect of BAGV in oviposition and life cycle of mosquitoes: *Culex quinquefasciatus* and *Cx. tritaeniorhynchus* mosquitoes (n=50 each) were infected orally with BAGV and allowed to oviposit. The number of egg rafts obtained, duration of oviposition, hatching rate and duration to complete the larval and pupal stages, duration of emergence and adult survival were recorded. Similar data from an equal number of uninfected adults were also recorded and analyzed to determine the effect of BAGV in mosquito survival and oviposition.

Establishment of persistent infection of BAGV in Cx. tritaeniorhynchus mosquitoes: *Culex tritaeniorhynchus* mosquitoes were inoculated with BAGV as described earlier[1], incubated for seven days and fed on normal infant mice. Fully engorged mosquitoes were collected, placed in double walled mosquito holding cages and allowed to lay eggs. Eggs were allowed to hatch and grow to adults. The eggs, larvae and adults were screened for presence of BAGV by tissue culture assay[1]. The mosquitoes were reared continuously upto 4th generation and used for experimental studies.

Results

BAGV yield in BHK-21 cell line: BAGV growth kinetics in BHK-21 cell line showed maximum yield of 5.5 and 5 $\log_{10}$ $TCID_{50}$/ml in cells and tissue culture fluid (TCF), respectively at 36 h PI. However, virus titre in TCF was maintained upto 84th h PI while a sharp decline in titre was detected in cells (Fig. 1). During

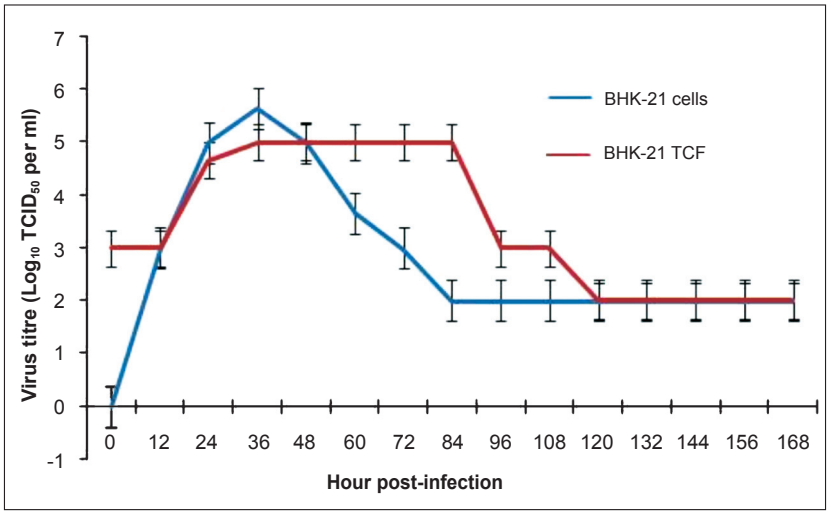

Fig. 1. Growth kinetics of BAGV in BHK-21 cell line and tissue culture fluid (TCF). Values are shown as mean ± SD (n=3).

stock preparation, cells were harvested at 60 h PI and the titre was determined as 5 $\log_{10}$ $TCID_{50}$/ml.

BAGV infection alters the replication of JEV/WNV during simultaneous infection: In the pilot study carried out by intrathoracic inoculation of *Cx. tritaeniorhynchus* mosquitoes, BAGV was found to play a significant role in inhibition of replication of both JEV and WNV (Fig. 2). In presence of BAGV, JEV replication was found affected both by simultaneous infection as well as in mosquitoes previously infected with BAGV. However, the influence was more prominent in the latter case as approximately 4 log reduction in virus titre was obtained both at 7th and 14th day PI (Fig. 2b) while the reduction in the simultaneous infection was only 2 $\log_{10}$ $TCID_{50}$/ml (Fig. 2a). JEV titre in mosquitoes on '0' day was approximately 2 log virus (100 pfu) in all the experiments. In the case of WNV replication in *Cx. tritaeniorhynchus*, simultaneous infection or super-infection with WNV yielded almost identical results (Fig. 2c, d) despite the initial titre of 2 log $TCID_{50}$/ml on '0'day in all the experiments. At 7th day PI, reduction in WNV yield was prominent (4 log) while on 14th day PI, the difference in WNV yield was approximately 2 $\log_{10}$ $TCID_{50}$/ml in either mode of infection.

Presence of BAGV did not alter the susceptibility of *Cx. quinquefasciatus* mosquitoes to JEV. In both the methods of infection, reduction in JEV yield in presence of BAGV was approximately 1 $\log_{10}$ $TCID_{50}$/ml only (Fig. 3a, b). No difference in virus yield was found on 14th day PI in simultaneously infected mosquitoes as identical titres were obtained in BAGV infected mosquitoes and normal mosquitoes (Fig. 3a) though a difference of 1 log was detected in mosquitoes previously infected with BAGV (Fig. 3b). However, susceptibility to WNV was found to be altered as remarkable reduction was observed in both simultaneously infected and mosquitoes previously exposed to BAGV (Fig. 3 c, d). The reduction in virus

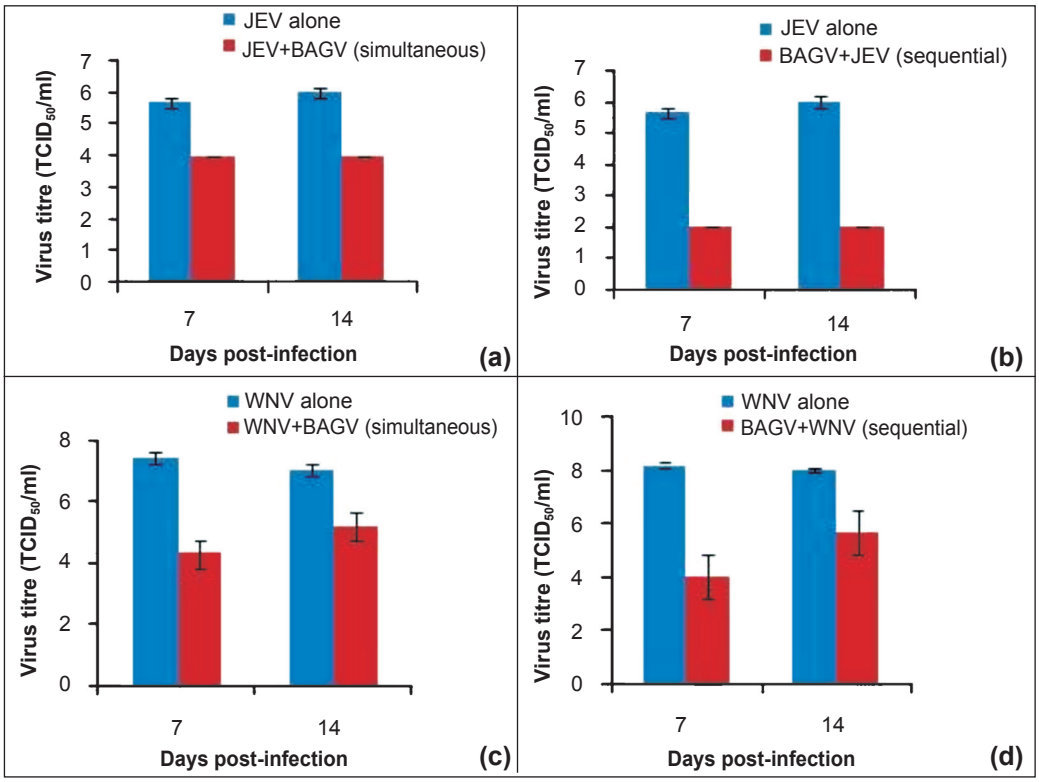

Fig. 2. Influence of BAGV infection in replication of JEV and WNV in *Cx. tritaeniorhynchus* mosquitoes (intrathoracic inoculation method); **(a)** Differential yield of JEV in mosquitoes infected simultaneously with BAGV to mosquitoes infected with JEV alone; **(b)** Differential yield of JEV in mosquitoes previously infected with BAGV to mosquitoes infected with JEV alone; **(c)** Differential yield of WNV in mosquitoes infected simultaneously with BAGV to mosquitoes infected with WNV alone; **(d)** Differential yield of WNV in mosquitoes previously infected with BAGV to mosquitoes infected with WNV alone. Values are mean ± SD (n=3).

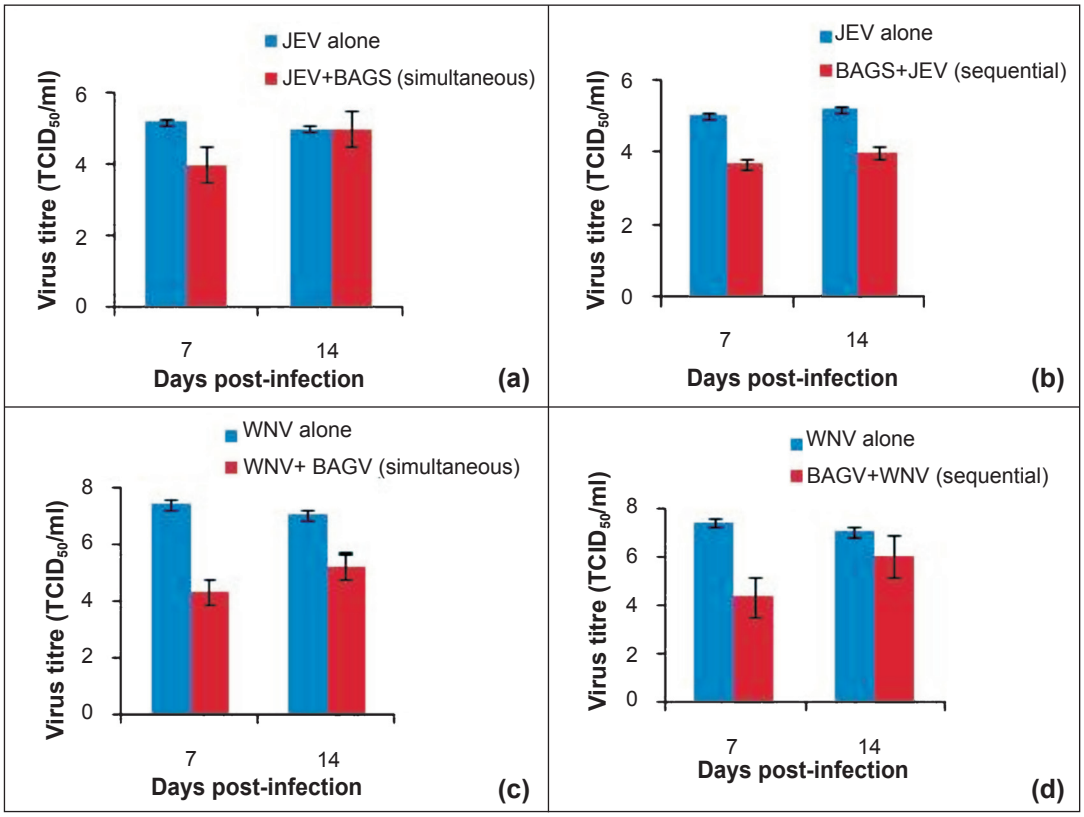

Fig. 3. Influence of BAGV infection in replication of JEV and WNV in *Cx. quinquefasciatus* mosquitoes (intrathoracic inoculation method); **(a)** Differential yield of JEV in mosquitoes infected simultaneously with BAGV to mosquitoes infected with JEV alone; **(b)** Differential yield of JEV in mosquitoes previously infected with BAGV to mosquitoes infected with JEV alone; **(c)** Differential yield of WNV in mosquitoes infected simultaneously with BAGV to mosquitoes infected with WNV alone; **(d)** Differential yield of WNV in mosquitoes previously infected with BAGV to mosquitoes infected with WNV alone. Values are mean ± SD (n=3).

yield was more prominent at day 7 PI than day 14 PI as the differences in titre of 3 $\log_{10}$ and 1$\log_{10}$TCID$_{50}$/ml were observed, respectively.

Effect of BAGV in orally fed mosquitoes: Oral feeding experiments were conducted only with JEV in *Cx. tritaeniorhynchus* and WNV in *Cx. quinquefasciatus* mosquitoes in presence of BAGV. Simultaneous infection of the viruses was not conducted in the study (oral feeding). In both species of mosquitoes, BAGV was found to be playing an important role in inhibiting JEV and WNV (Fig. 4). JEV replication was found restricted to approximately 2 $\log_{10}$ TCID$_{50}$/ml in BAGV infected mosquitoes upto 9th day PI in comparison to JEV yield in normal mosquitoes which yielded approximately 5 $\log_{10}$ TCID$_{50}$/ml during the same period (Fig. 4a). However, from 9th day PI onwards, enhanced replication of JEV was observed yielding titres comparable to JEV yield in normal mosquitoes. In *Cx. quinquefasciatus* mosquitoes the influence of BAGV was found prominent in inhibiting WNV replication upto 15th day PI (Fig. 4b). The difference in virus yield was prominent during 5th to 13th day PI which showed 2-3 $\log_{10}$ TCID$_{50}$/ml reduction in WNV titre in comparison to WNV yield in the controls.

Establishment of persistent infection of BAGV in Cx. tritaeniorhynchus mosquitoes and their susceptibility to JEV: Persistent infection of BAGV was established in *Cx. tritaeniorhynchus* mosquitoes by serial passaging of infected mosquitoes. Presence of BAGV was detected in eggs (1.7 $\log_{10}$ TCID$_{50}$/ml) laid by the 3rd generation of mosquitoes. The progeny of the 4th generation adults, when infected with JEV showed a gradual decline and maintained at a titre of 2 $\log_{10}$TCID$_{50}$/ml upto 9th day PI demonstrating a reduction of approximately 3 $\log_{10}$TCID$_{50}$/ml in comparison to JEV yield in normal mosquitoes (Fig. 5). However, an increase in JEV titre to approximately 5 $\log_{10}$ was detected on 11th day PI, which was maintained upto 14th day PI.

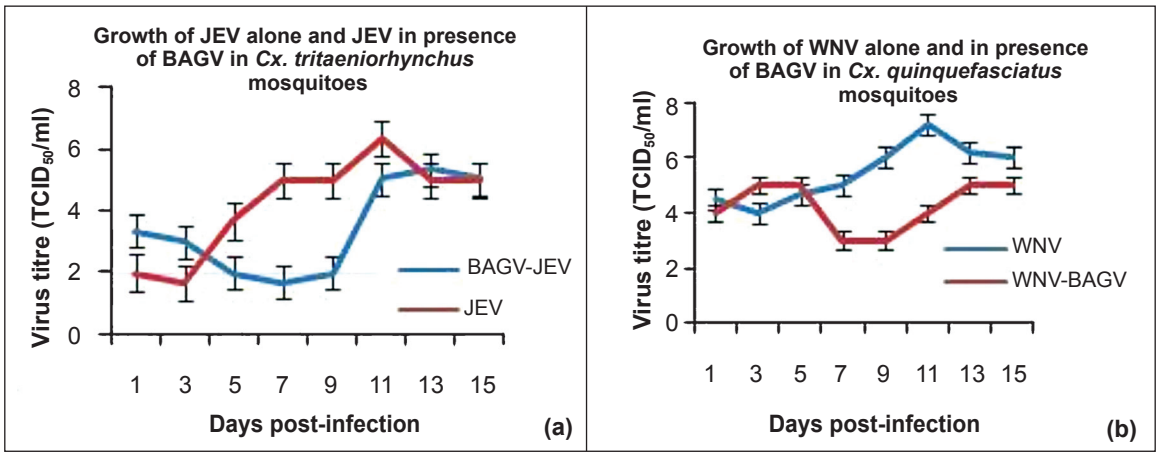

Fig. 4. Influence of BAGV infection in replication of JEV and WNV in *Cx. tritaeniorhynchus and Cx. quinquefasciatus* mosquitoes (oral route). Values are mean ± SD (n=3).

Role of BAGV in the natural cycle of Cx. tritaeniorhynchus mosquitoes: During the establishment of persistent infection, it was observed that egg laying and hatching of eggs was affected by BAGV. When a virus titre of 4.7 $\log_{10}$ $TCID_{50}$/ml was used to infect mosquitoes by oral feeding, only three egg rafts could be obtained from 43 fully engorged mosquitoes. However, none of the eggs hatched. Eggs were retained in the ovaries of the infected mosquitoes. In subsequent experiments, the virus concentration was lowered 10- and 100-folds which resulted in higher number of egg rafts (Table). However, the number of eggs laid was comparatively lower than what was obtained in normal mosquitoes (Table).

Discussion

In the present study, we explored the potential of BAGV in influencing replication of JEV and WNV in *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes. Earlier studies have shown that mosquito cell line or a susceptible mosquito, once infected with an arbovirus, does not get infected from subsequent infection with another virus of the same group[10-17]. Similar phenomenon has also been observed in mosquito cell lines infected with alphaviruses[18]. It was observed that mosquito cell lines persistently infected with Sindbis virus altered the productivity of other togaviruses in the cell lines upon subsequent infection. Hobson-Peters *et al*[19] demonstrated suppression of WNV and Murray Valley fever encephalitis viruses in mosquito cell cultures infected with Palm Creek virus, a new flavivirus isolated from *Coquillettidia xanthogaster* mosquitoes. Contradictory to the above findings, Kent *et al*[20] could not demonstrate suppression of WNV replication in *Cx. quinquefasciatus* mosquitoes infected with the insect flavivirus, *Cx. flavivirus* Izabel. They could not find any significant impact either on virus replication or transmission of WNV in the mosquitoes previously infected with the mosquito flavivirus. Rather, they observed enhanced WNV transmission in a certain population of *Cx. quinquefasciatus* mosquitoes upon simultaneous infection.

In the present study using intrathoracic inoculation of mosquitoes, suppression of replication of both JEV and WNV was observed in mosquitoes on 7th and 14th day PI either infected simultaneously or sequentially. These findings demonstrated partial super-infection exclusion in *Cx. tritaeniorhynchus* infected sequentially with BAGV rather than simultaneous infection. Unlike JEV, significant reduction in WNV titre was observed in both *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes in presence of BAGV especially on 7th day PI.

In the oral feeding experiment, which is the natural route of infection, the findings were almost identical to that of inoculation method. Partial exclusion was observed with JEV and WNV in *Cx. tritaeniorhynchus* and *Cx. quinquefasciatus* mosquitoes, respectively. Similar results were obtained with BAGV persistent mosquitoes also. This has significance in nature as there is a possibility of mosquitoes being infected with different insect viruses and these viruses might be playing an important role in the control of pathogenic viruses[21].