June 2009 Volume 3 Issue 3

Trade Science Inc.

BioTechnology

An Indian Journal

FULL PAPER

BTAIJ, 3(3), 2009 [174-178]

Comparative susceptibility of BHK-21 and vero cell lines to bluetongue virus (BTV) isolate pathogenic for sheep

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Received: 29th April, 2009 ; Accepted: 4th May, 2009

ABSTRACT

Bluetongue (BT) is an infectious, non contagious arthropod-borne disease of ruminants caused by Bluetongue virus (BTV), prototype species of the genus *Orbivirus*, within the family *Reoviridae*. In Tamil Nadu, 22 out of 24 districts were reported to be affected by the bluetongue virus. Hence the attempt has been made to cultivation and adaptation of BTV isolates in two different cell lines namely BHK 21 and Vero. The study was found to be successful as the inclusion bodies induced by the virus (intra cytoplasmic eosinophilic inclusions, nuclear fusion with giant cell formation). It was evident from the infectivity titre of 9.48 that the virus has got adapted to BHK21 cells to a greater extent than Vero cells as it happened to be an ideal cell line for Bluetongue vaccine production. The silent persistent infection of the BHK 21 and Vero cell lines cells with BTV indicates that more stringent screening of the cells used in the production of live vaccines.

KEYWORDS

BTV;
BHK 21;
Vero cell lines;
CPE.

INTRODUCTION

Bluetongue (BT) is an infectious, non contagious arthropod-borne disease of ruminants caused by Bluetongue virus (BTV), prototype species of the genus *Orbivirus,* within the family *Reoviridae*. Twenty four serotypes of bluetongue virus have been identified till date[5].

India has significant population of domestic and wild ruminants which are known to be susceptible to bluetongue virus infection. Several exotic breeds of sheep were introduced into the country between 1960 and 1970 for the genetic improvements of national flock by cross breeding with native breeds. In India, the total sheep population is 51 million, accounting to 5 percent of world's sheep population and 123 million goats accounting for 20 per cent of the total global livestock[6].

In Tamil Nadu, 22 out of 24 districts were reported to be affected by the bluetongue virus. The reported case of bluetongue virus among sheep and goats occurs presumable in an epidemic form during the South-west monsoon season which favours vector population of bluetongue virus[10]. Although the history of reporting was not continuous, the number of outbreaks, attacks and deaths among ruminants reported is of great concern that needs immediate attention for the protection of livestock and economic growth[19].

The development of laboratory based systems for the investigation of animal disease such as bluetongue is crucial to understand the infectious agent and the dis-

ease process globally. As a result, adaptation of bluetongue virus to grow in laboratory conditions, such as cell culture techniques. It has been possible to develop a range of diagnostic tests and vaccines[3]. Hence the attempt has been made to cultivation and adaptation of BTV isolates in two different cell lines namely BHK 21 and Vero.

MATERIALS AND METHODS

Bluetongue virus isolation

One hundred and sixty five (165) blood samples were collected from BTV infected animals of each district of Coimbatore, Dindigul, Erode, Karur, Namakkal and Salem. The blood samples were collected from animals in their high febrile period by jugular vein puncture in a vaccutainer tubes containing EDTA[21]. The collected samples were kept in vaccine bath and transported to the laboratory. For long-term storage the blood samples were collected in a vial containing Oxalate-phenol -glycerine (OPG) and stored at -70^0C[15].

Cultivation of BTV isolates in BHK 21 and vero cell lines

Cell lines

Established cell lines such as BHK-21 and Vero were obtained from National Centre for Cell Sciences, Pune, India. The cell lines were sub-cultured and maintained in minimal essential medium (MEM).

Propagation of cells

Cells were diluted in growth medium so that it contained 1×10^5 cells/ ml and distributed in tissue culture bottles. For each tissue culture bottles, 5ml of growth medium (10%FBS) was added. The seeded bottles were incubated at 37^0C, 5% Co_2 with humidity till the monolayer was completed. After 48 hours of incubation, the growth medium was discarded and 5ml of maintenance medium was added and tissue culture bottles were incubated at 37^0C, 5% Co_2 with humidity over night[20].

Inoculation of virus into cell line

The blood samples collected and separated the RBC and lysed with distilled water. The lysed RBC was clarified by centrifugation at 10,000 rpm for 10 min at 4^oC. The virus in the supernatant was filtered and 0.1 ml of viral sample was added (0.22µm) to baby hamster kidney BHK-21, and Vero cell lines using syringe filter[19]. The efficiency of isolation was often significantly lower following direct addition to cultured cells compared with that achieved in ECE. Greatest efficiency of isolation in cell culture is achieved by first passaging ECE homogenates in cells, followed by either antigen detection procedures or additional passages in mammalian cell lines, such as BHK-21 and Vero cytopathic effect (CPE) was necessarily observed in cells. Cell monolayers were monitored for the appearance of a CPE for 5 days at 37^0C in 5% Co_2 with humidity. Cytopathic effects show the presence of inclusion bodies in the intracytoplasamic of bluetongue virus infected BHK-21 cell line[2].

Detection of cytopathic effect

After 48 hours the tissue culture bottle were observed for cytopathic effect, like cytopathic changes/ morphological changes using inverted microscope. CPE occurs in a uniform manner in the tissue culture bottles. The range of percentage of CPE is detected by using dilution virus titre method[16].

Virus titration in microtiter plate[16]

Log dilution of virus to be titrated was prepared. 4.5ml of HBSS were added to sterile test tubes and 0.5ml of virus suspension was added to the first tube. It was mixed gently and 0.5ml was transferred to the next diluent tube successively. It was mixed and added using a fresh pipette to the subsequent dilution without touching the diluent and the pipette was discarded after transfer of the 0.5ml volume. 50µl MEM was distributed to 7 rows of 8 wells sterile microtitre plate by using a multichannel pipette. 50µl of each virus dilution was added to appropriately marked rows. 100µl of cell suspension was added in growth medium with 10% fetal calf serum. The microtitre plates were incubated for observing CPE.

RESULTS AND DISCUSSION

The blood samples were collected from the infected sheep for the isolation of BTV. In the four year study period there were 95 field outbreaks from which 165

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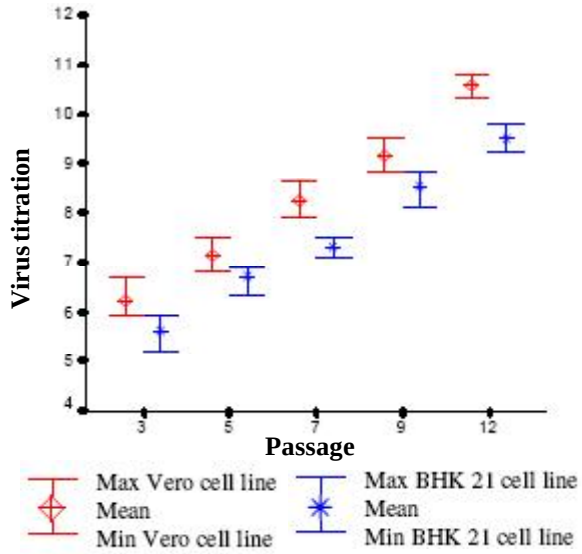
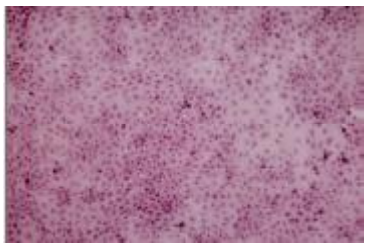


Figure 1: Comparison of virus titration in two cell lines

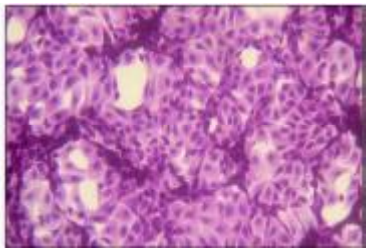
samples were collected for the isolation purpose. The virus was cultivated in two different cell cultures like BHK21 and Vero to find out its susceptibility and production of high titre. In this study, infectivity virus titration was done in Vero cell line and BHK 21 cell line and their findings are summarized in **TABLE** 1, and figure 1. In BHK 21 and Vero cell lines the BTV isolates produced visible CPE from 36h post infection, infected cells became swollen and well defined. Rounding of cells, syncytia formation, giant cell formation and grouping of cells have also been observed in fifth passage. The affected cells have shown granularity and undergone variable degrees of shrinkage. Later, most of the cells got detached from the glass (Plate 1 and 2).

Infectivity titre values of the thirteen isolates ranged between 6.21 and 10.58 and 5.6 and 9.48 from 3rd to 12th passage in the case of Vero cell line and BHK 21 respectively. Between the two cell cultures, the Vero cell line exhibited more titre value than BHK 21 (TABLE 1). This was significantly noticed in the mean difference values (p=<0.0001). Significant difference was observed right from the 3rd passage to 12th passage in all the thirteen isolates in terms of mean infectivity titre value (Figure 1) and minimum and maximum titre value. It can be statistically concluded that Vero cell line exhibited maximum infectivity titre than BHK21 cell line.

Bluetongue has been recognized as an economically significant vector borne, non contagious, viral disease of domestic and wild ruminants in many parts of



A- Untreated Vero cell line (40x Magnification)



B- Bluetongue infected vero cell line (40x Magnification) (48h post infection)

Plate 1 : Cytopathic effect of Bluetongue virus on vero cell line stained with Giemsa

A- BHK -21 cell line uninefected (40x Magnification)

B- Bluetongue infected BHK-21 cell line (40x Magnification) (48h post infection)

Plate 2 : Showing cytopathic effect of bluetongue on BHK - 21 cell line stained with Giemsa

the world[11]. Bluetongue continues to be a major disease affecting sheep in all countries in the tropics and subtropics including India. The economic loss due to bluetongue outbreaks in sheep is very high. Control of the disease is very difficult because of the plurality of the serotypes that occur and no single vaccine may be effective[13]. In the absence of vaccines available for the control of Bluetongue virus, the other measures are needed to be adopted such as control of the vector

population, treatment of animals for secondary bacterial infection and nursing of the animals. These control measures would be worthwhile if early diagnosis of the disease is accomplished[14].

In BHK 21 and Vero cell lines, the BTV isolates produced visible cytopathic effect (CPE) from 36h post infection. Infected cells became swollen and well defined. Rounding of cells, syncytia formation, giant cell formation and grouping of cells have also been observed in fifth passage. The affected cells have shown granularity and undergone variable degrees of shrinkage. Later, most of the cells got detached from the glass. The same type of CPE and changes in the cell morphology was observed by Meenambigai et al.[12], who made a comparative study of the inclusion bodies in BHK 21 and Vero cells infected with BTV. During initial passages there were more refractile cells in the cell monolayer, with aggregation and clumping of cells in 48 hours post infection in both cell lines. The initial stage of infection of cellular destruction coupled with clumping of cells and formation of polykayotes were noticed. Later, rounding of cells became more prominent with syncytia formation. The pores in the nuclear envelope appeared much enlarged in BHK21 infected cells than Vero infected cells as compared with the control cell culture preparation. This was in accordance with the findings of earlier research workers Nachimuthu et al.[13] and Clavijo et al.[4].

In our study, BHK-21 cells have shown a high sensitivity to BTV infection, producing a distinct cytopathic effect (CPE) at 3-5 days post-infection. The CPE is characterized by the appearance of foci of rounded and refractile cells. These foci rapidly enlarged to the point of rupture, whereupon they detach and float freely in the culture media. However, as reported by Afshar[1], isolation of BTV from field samples required the amplification of BTV in ECE before tissue culture can be used. If direct inoculation in tissue culture of field samples is required, this should be carried out in parallel with ECE inoculation[1].

The infectivity titres of BTV isolates in BHK 21 and Vero cell lines were adduced at the end of 3rd, 5th, 7th, 9th and 10th passage levels. A gradual rise in titres was observed with respect to all the isolates as the passage levels increased. At the end of 12th passage the (Log_{10}) tissue cultures infective dose 50 (TCID50) was found to be between 10.3 and 10.8 in Vero cell line and 8.1 and 8.8 in BHK 21 cell line ($p<0.0001$). It housed significant difference in the each passage titre value. In the earlier study of Reed and Muench[16], a titre value of 7.5 and 6.5 was observed in BHK21 and Vero infected cells at 15th passage. These findings are in agreement with the earlier observations made by Howell and Verwoerd[9] and Scott et al.[17].

The various mammalian cell lines, Baby Hamster Kidney (BHK21), African green monkey kidney (Vero) and an insect cell line (C6/36) derived from *Aedes* species were the most common types used for BTV propagation[18]. BHK21 and Vero cells offer a way of growing BTV to comparably high titres, free of cell metabolites[8] and hence was chosen in the present study for the adaptation of BTV. Generally the use of cell cultures for the primary isolation of BTV from the field samples is not recommended. Routinely, it is necessary to amplify the virus in ECE before growing in cell culture for eventual identification using serological assays for viral antigen production[7]. The same principle was followed in the present study and the outcome of the study was found to be successful as the inclusion bodies induced by the virus (intra cytoplasmic eosinophilic inclusions, nuclear fusion with giant cell formation) correlated with the findings of earlier workers (Brown and Jochim 1962). Moreover, it is also evident from the infectivity titre of 9.48 that the virus has got adapted to BHK21 cells to a greater extent than Vero cells as it happened to be an ideal cell line for Bluetongue vaccine production.

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CONCLUSIONS

The occurrence of BTV disease in India became endemic in native breeds. In Tamil Nadu there are 58 lakhs of sheep in which 12 lakhs are Macheri breed. In 2005 around 2 lakh sheep were suffering from BT disease, because no of serotypes of BTV causes failure vaccine production. The BTV were cultivated in two mammalian cell lines such as BHK21 and Vero. The BHK 21 cell lines were produces high infectivity titre i.e., 9.48 that the virus has got adapted to BHK21 cells to a greater extent than Vero cells. The silent persistent infection of the BHK 21 and Vero cell lines cells with BTV indicates that more stringent screening of the cells used in the production of live vaccines.

ACKNOWLEDGMENTS

The authors are thankful to the Principal and the Management, K.S.R. College of Educational Trust, Tiruchengode, Tamilnadu, India, for providing necessary facilities and constant encouragement to carry out this study.

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