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Evaluation and concentration of polyethylene glycol assay for early diagnosis of Dengue antigen 2

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ABSTRACT

Dengue, a mosquito borne viral infection in recent years is been a major global public health problem with increasing number of reports of *Dengue* fever and *Dengue* hemorrhagic fever with usual manifestations every year. In spite of availability of various diagnostic methods including high sensitive molecular techniques, WHO recommends, 'Haemagglutination-inhibition (HAI)' over expensiveness and non-feasibility of others. Therefore there is a need for time saving and in expensive method for concentration. This report conveys that polyethylene glycol (PEG) for concentration of *arboviruses* had been used for rapid diagnosis of *Dengue* type II viruses. In this study two methods using PEG were evaluated for concentration and are recommended as an initial step in the concentration of *arboviruses* propagated in tissue culture, as it is simple, rapid, and inexpensive.

Key words: Polyethylene glycol (PEG), Haemagglutination-inhibition (HAI), *Dengue* type II viruses, *Arboviruses,* World health organization (WHO).

INTRODUCTION

Dengue viruses belong to the *flavivirus* and family *flaviviridae*. The *Dengue virus* is classified into four antigenically distinct serotype namely *D1, D2, D3* and *D4*. *Dengue* is currently the most important arthropod borne because over past two decades there has been a dramatic global increase in the *Dengue* Fever (DF), *Dengue* hemorrhagic Fever (DHF) and *Dengue* shock syndrome (DSS). *Dengue* spread by *Aedes mosquitoes* (*Aedes aegypti* and *Aedes albopictus*) [1]. The world health report 1996 stated, that the "re-emergence of infectious disease is a warning the progress achieved so far towards global security in health and prosperity may be wasted" [2].

Dengue infection frequently occurred in children and infection in adults frequently produces symptoms. Some virus strains, however, produce very mild illness in both adults and children, which is often not recognized as *Dengue* and circulates silently in the community [3]. The first evidence about the occurrence of *Dengue* fever in this country was reported during 1956 from Vellore district in Tamilnadu. The first outbreak occurred in Calcutta (West Bengal), in which 30% of cases showed hemorrhagic manifestations. Delhi, situated in the northern part of India, had outbreak of *Dengue virus* infection due to different *Dengue virus* types in 1967, 1970, 1982 but no culture confirmed cases of *Dengue* Hemorrhagic Fever and *Dengue* Shock Syndrome (DHF/DSS) were reported during this epidemic. These were confirmed only by haemagglutination inhibition test. In 1996, one of the most severe outbreaks of *Dengue* Hemorrhagic Fever and *Dengue* Shock Syndrome (DHF/DSS) occurred in Delhi, where in 10252 cases and 432 deaths occurred [4]. Many outbreaks have been reported since 1965 in Vellore, with fairly recent in 1997 from Chennai and more recently in 2007. The outbreaks in India occur mostly during or after rainy season, which coincide with the rise in the vector population.

Isolation of *Dengue virus* from female *Culex tritaeniorhynchus* was performed by inoculation of specimens into, *Aedes albopictus* clone C6/36 cells as well as into suckling mouse brain (SMB) [5]. A method for isolations and identification of *Dengue viruses* is described [6]. Viruses were isolated mosquito cell cultures (C6/36) or (Ap-61), identified by indirect florescent antibody technique and typed by complement fixation test, using the cell culture fluid as an antigen. Incubating the mosquito cells at 32°, *Dengue* viruses can be identified within 6 days after incubation. In comparative titration of *Dengue* viruses in C6/36 and Ap-61 cells, the C6/36 cell lines gave generally higher titers [6]. Isolation of *Dengue viruses* in *Aedes albopictus* cell cultures belonging to all four serotypes could be isolated directly from human sera and mosquito suspensions. The isolation and identification of *Dengue viruses*, probably by enhancing the infective virus titers [7, 8]. Isolation of a Singh's *Aedes albopictus* cell clone sensitive to *Dengue* and *Chickungunya viruses*. Growth curve of the viruses indicated that clone C6/36 gave a significantly higher yield for each viruses than uncloned SAAR cells up to 7 days after infection [5].

Thus the serological and molecular methods are highly sensitive but cannot be performed in all laboratories. The kits available for ELISA are expensive and the antigen requirement for HAI is high [9]. Therefore there is a need for time saving and in expensive method for concentration of *Arboviruses.* A simple method for concentration of *arboviruses* propagated in tissue culture by using polyethylene glycol (6000 M). Concentration of four serologically unrelated *arboviruses* with this method showed that recoveries compared well with those obtained after ultra centrifugation. The method is recommended as an initial step in the concentration of *arboviruses* propagated in tissue culture, as it is simple, rapid, and inexpensive. In this study two methods using PEG were evaluated for concentration of *Dengue* type II viruses.

MATERIAL AND METHODS

Preparation of mouse brain antigen

Four sets (a set contains 5 miceses) of 1 to 2 days old suckling mice (Swiss albino) were inoculated intracerebrally with 0.02 ml *Dengue*$_2$ *virus* [10]. The mice were observed for morbidity and mortality for 7 to 10 days. After paralysis and death, the brain tissue was harvested, weighed and four volumes 8.5 % aqueous solution of sucrose was added. The brain tissue was homogenized for three-1 minute cycles. The entire operation was carried out in an ice water bath. The homogenized mouse brain solution was expelled thorough and 18-gauge needle into a container of acetone (one volume of homogenate to 20 volume of acetone). The suspension was mixed, shaken vigorously and held at 4° for 1h. Suspension was centrifuged at 2000 rpm for 10 min. The acetone was decanted carefully and the sediment was dried for 1 h at 4°. To the dried homogenate, 0.9 % normal saline (2/5of the volume of original homogenate) and a few glass beads were added. The contents were shaken for a few minutes and then placed at 4° overnight for complete rehydration. The suspension was then centrifuged at 12000 rpm for 1h. The supernatant fluid was collected and stored at -70° for further use. Approval for use of mice for antigen preparation was obtained from the institutional ethical committee according to national guidelines.

Collection and washing of Goose erythrocytes

Erythrocytes from adult white domestic goose (*Anser cinereus*) were used for haemagglutination and haemagglutination inhibition procedures. 7.5 ml of acid citrate dextrose was taken in a syringe fitted with a 20- gauge needle. About 50 ml of venous blood was collected from the wing of the vein. The blood was transferred to a flask containing 125 ml of alsever's solution. The suspension was mixed and stored at 4°. The required quantity of the erythrocyte suspension was taken in centrifuge tubes and centrifuged for 6 min at 2000 rpm. The supernatant fluid and the buffy layer of white blood corpuscles were removed without disturbing the packed erythrocytes. 2 ml of normal saline was added to the packed erythrocytes and mixed to get a complete resuspension. Additional quantity of normal saline was then added to restore the original volume. The suspension was then centrifuged at room temperature for 3 min at 2000 rpm. The above steps were repeated. The supernatant fluid was removed and sufficient quantity of saline was added based upon the volume of the packed RBC in order to make a 10% suspension.

Haemagglutination activity

The haemagglutination activity of the *arboviruses* is pH dependant. Therefore it is necessary to titrate the antigen in different pH solution. This is useful to determine the optimum pH and the antigen dilution to be used[11]. 0.05 ml of Bovine albumin in borate saline (BABS) was added from the second well of each row and the micro titer plate was chilled in a refrigerator for a few minutes, 0.05ml of BABS was added to the cell control wells and 0.1 ml of the neat antigen was added to the first and second wells of each row in a plate. 2.4 ml of the Viral Adjusting Diluents (VAD) of the pH ranging from 6.0, 6.2 and 6.4 were taken in labeled tubes. 0.4% of RBC was prepared using 10% goose RBC stock solution. 0.05 ml of the prepared erythrocytes was added to the cell control wells. A plate shaker was used to mix the cells and antigen dilutions. The plate was then sealed and incubated at 37°C until the cells in the controls settled and formed a clear button. The titration was recorded; the cell control well showed a clear button. Agglutination is indicative of positive HA result and button formation is indicative of negative HA result.

Complete agglutination consists of a layer of uniformly agglutinated cells covering the lower surface of the well. A negative pattern consists of a compact, sharply demonstrated button of sediment cells in the centre of the well, the pattern being identical to that seen in the control wells. The highest dilution of antigen with complete agglutination is the titre of the antigen and contains one unit of antigen. The virus adjusting diluents with which the antigen shows the highest titre is the optimum pH and should be used for that particular antigen.

Virus Concentration by Poly Ethylene Glycol (PEG) Preparation

The concentrations of viruses by Poly Ethylene Glycol (PEG) were evaluated for Mullins laboratory method by one volume of PEG (6000 M) solution was added to four volumes of viruses. It was refrigerated overnight at 4° and centrifuged at 2300 rpm for 45 min and the supernatant was removed. The virus pellet was resuspended in 1/10 and 1/100 of original volume and the evaluation of Marian Horzinek's method[12] by employing equal volume of PEG (6000 M) was added to the antigen and mixed well at 4°C. After that it was centrifuged at 8000 rpm for 30 min. Then the pellet was resuspended with the buffer. This elution was allowed to proceed for 60 min at 4°C under continuous stirring and followed by the haemagglutination test was carried.

RESULTS AND DISCUSSION

Dengue antigen is prone to drop in titer when stored for a period of time or when freeze thawed. There is a need for development of a concentration protocol and to find a stabilizer to maintain the titer. In this study two concentration methods were followed by Mullins lab method and Methods described by Marian Horzinek were evaluated (Table 1).

In this study two methods using PEG were evaluated for concentration of *Dengue* type II Viruses. Of the two methods used it was observed that the method described by Marian Horzinek was found to be the most effective. The final volume was 1/10th of the initial volume and the titer was increased 4 times that is 1/256 (Table 2).

Table 1: Viral antigen pellet was dissolved in 1/10th and 1/100th for the starting volume of Mullins lab method were obtained as 1:64 and 1:128 titers respectively, whereas 1/10 reconstituted antigen of Marian Horzinek showed titer of 1: 256

Evaluation of arbo viral antigen concentration by Mullins lab method and methods described by Marian Horzinek				
Concentration methods	**Initial**	**Concentration**		
	Volume	**Titer**	**Volume**	**Titer**
Mullins lab method - 1/10 dilution	2ml	1:64	200µl	1:64
Mullins lab method -1/100 dilution	2ml	1:64	20µl	1:128
Marian Horzinek - 1/10 dilution	2ml	1:64	200µl	1:256

Table 2 The final volume for Marian Horzinek was 1/10th of the initial volume and the titer was increased as 1/256

Marian horzinek – arbo virus concentration by 1/10 dilution				
	Initial		Concentration	
Days	Volume	Titer	Volume	Titer
After 10 Days	2ml	1 : 64	200 µl	1:256
After 20 Days	2ml	1 : 64	200 µl	1:256
After 30 Days	2ml	1 : 64	200 µl	1:256

Table 3: Antigen stability was checked for a month and the Marian described method is increasing the antigen titer and the final volume is also significantly more compared with the Mullin method

stability of mice brain antigen by direct haemagglutination		
Days	Initial	
	Volume	Titer
After 10 Days	2 ml	1:32
After 20 Days	2ml	1:32
After 30 Days	2ml	1:16

The Mullins method was employed for the same volume of starting sample and the pellet was dissolved in 1/10th and 1/100th of the starting volume. When the pellet was reconstituted in 1/10th the titer obtained was 1:64 whereas the 1/100 reconstituted antigen showed 1:128 titers (Table 1). Though the tire was increased final volume was very less therefore this method cannot be recommended.

The Marian described method is not only increasing the antigen titer, the final volume is also significantly more compared with the Mullin method and the time taken is also very less. Therefore Marian method can be used for concentration of *arboviruses*. Antigen stability was checked for a month (Table 3). Ten days once haemagglutination was put up to asses the antigen titer stability. Stability maintained in the concentrated antigen. This antigen could be lyophilised and used for further research purpose.

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