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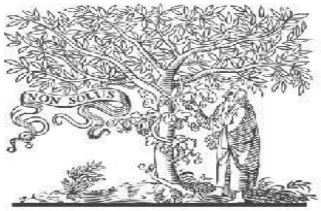

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Paper Authors **KOMMU VENKATA BIXAMAIAH, Dr. Kapil Kumar**

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A STUDY ON NUCLEIC ACID AMPLIFICATION OF SEROTYPING FOR DENGUE VIRUS

CANADIDATE - KOMMU VENKATA BIXAMAIAH
DESIGNATION- RESEARCH SCHOLAR MONAD UNIVERSITY DELHI HAPUR
GUIDE NAME- Dr. Kapil Kumar
DESIGNATION- PROFESSOR MONAD UNIVERSITY DELHI HAPUR

ABSTRACT
The relevance of the effective and the most accurate diagnosis of dengue is essential for the proper analysis of clinical sample, its surveillance activity, outbreak control, pathogenesis, academic research, and other trails. Several clinical tests can be performed to identify and detect the viral load, the antigen-antibody reaction, and viral nucleic acid. Nucleic acid detection is the most sensitive and specific method of Dengue diagnosis. Viral nucleic acid appears earlier than antibodies so the infection can be detected at very early stage. This is an advance method by which further research such as molecular characterization of infecting virus can also be done. However, this method is expensive, needs technical expertise and advanced laboratory set up. Several other nucleic acids amplification-based techniques have also been developed to detect Dengue infection such as Loop mediated amplification methods and NASBA. Nucleic Acid Sequence Based Amplification (NASBA) does not require thermo-cycler. After the cDNA synthesis the newly formed double stranded DNA serves as a template for RNA transcription. The amplified RNA is detected by electrochemiluminescence or in real-time with fluorescent-labelled molecular beacon probes.
KEYWORDS: - nucleic acid, serotyping of Dengue virus, antigen-antibody reaction

INTRODUCTION

The mosquito breeding sites are known to be close to the domestic areas due to the use of non-biodegradable plastics which are left in open and storing water in the plastic bottles. Aedes aegypti being a tamed being is known to feed in each gonotrophic cycle mare than once and covers approximately 100m in the urban areas. Due to its sensitive nature to the slightest movement, the aegypti species intrudes its feeding several times and it takes blood meals from individual to complete its meal. It is known to be a principal dengue fever vector. It is broadly widespread all over the India. Along with this, the dengue virus infection was confined to urban areas and was recently detected in Aedesalbopictusand then later found its spread to rural areas with the areas that were free from the infection. In India, Dengue fever (DF) and Dengue hemorrhagic fever (DHF) are the major concern for public health reported in more than 19 states (Sharma, 2011; Negi et al., 2020). Dengue genome distribution in India from the year 2011-2017 with the help of the Indian partial sequences.

Within three to fourteen days after infection, the normal clinical symptoms are generally observed, with respect to that the recovery takes 2-7 days. The life-threatening dengue hemorrhagic fever led to bleeding which is ultimately plasma leaks and low levels of blood platelets else into critically low levels of blood pressure

which is referred to as dengue shock syndrome.

Dengue is a mosquito borne viral disease and the epidemiology depends on the climate, environment, and human activities. In equatorial and tropical monsoon zone, the conditions are suitable for the mosquito survival. The man-made environment also provides a suitable habitat for breeding. International travel has supported the transmission of the virus among countries. After the epidemic of Philippines and Thailand in late sixties, Dengue spread rapidly all over the world. The disease is now endemic in all continents except Europe, where the threat of outbreaks has started to appear. In 2010, Dengue cases were reported for the first time in Croatia, France and some other European countries. In 2012, an outbreak was reported in Portugal with over 2000 cases. Dengue is endemic in many Latin American and Asian countries. Around 3900 million people in 128 countries live at the risk of Dengue infection. Dengue endemic areas include more than 100 countries in Africa, the Americas, the eastern Mediterranean, south-East Asia and the eastern Pacific. The most affected areas are South America, south-east Asia and western Pacific regions. In the year 2013, over 3 million cases were reported from South and North America, south-east Asia and western pacific (WHO, 2016).

LIFE CYCLE

Aedes aegypti is a tropical and sub-tropical species. It is widely distributed around the world between 35˚N and 35˚S. It has been found as far as 45˚N. In these areas the mosquito invades during summers but it is unable to survive the winters. The species is relatively uncommon above 1000 meters. The average life span is not more than six weeks. The population fluctuates with rainfall and water storage because life span of Aedes aegypti is influenced by temperature and humidity. It survives best between 16º-30º C and a relative humidity of 60-80% and deposits its eggs in clean water. Eggs are very resistant to unfavourable environment and remain viable for several months in absence of water. In optimum conditions the eggs hatch in 3 days. The adult emerges 8-15 days later. These mosquitoes do not fly far and they spread over vast distances by means of manmade transportation. These are found in both indoors and outdoors and feed almost entirely on humans during day hours. The peak time of mosquito biting activity is during early morning for 2-3 hours after day break and in the afternoon. The female mosquito is very sensitive feeder and disrupts the feeding process even at the slightest movement. To complete one full blood meal the mosquito has to bite multiple persons. If infective, this mosquito transmits the virus to many individuals at the same feeding episode. That is why Aedes aegypti is considered an efficient vector of Dengue epidemic

TRANSMISSION CYCLE

After the bite of an infective mosquito, there is an Intrinsic Incubation Period (IIP-incubation period in humans) of 3-14 days (average 4-7 days), after which the individual may show acute fever with non-specific symptoms. During this acute febrile illness the individual is viremic and remains so for 2-10 days A mosquito which bites the person at this stage becomes infected and after an Extrinsic Incubation Period (EIP-incubation period

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in vector) of 8-12 days the mosquito becomes infective and can transmit the virus to other individuals it bites. Transmission cycle of Dengue virus between humans and mosquitoes is shown in Figure 1. Vertical transmission of Dengue virus from infected female mosquitoes to the next generation through eggs, known as trans-ovarian transmission also occurs between mosquito generations.

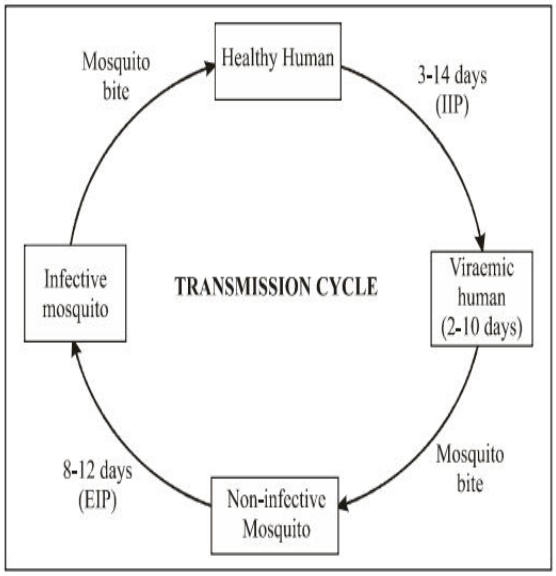
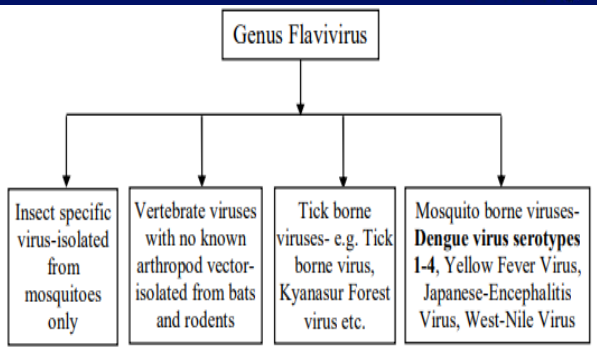


Figure 1. Transmission cycle of Dengue virus between Humans and Mosquitoes

DENGUE VIRUS

Dengue virus belongs to the family Flaviviridae and genus Flavivirus which contains approximately 70 viruses. These are 40–50 mm in size and spherical in shape with a lipid envelope. The genome is approximately 11,000 bases long and comprised of three structural and seven non-structural proteins. All viruses share common group epitopes on the envelope proteins resulting in extensive cross reactions in serologic tests. The genus Flavivirus can be divided in four sub-groups as shown in the flow chart given below.



Dengue virus is roughly spherical in shape and 50 nm in size. Two structural proteins, the Envelope (E) and Membrane proteins (M) are inserted in the lipid membrane. 180 identical copies of the Envelope (E) protein are attached to the surface of the viral membrane by a short transmembrane segment. Structure of Dengue virus, Envelope protein (E), Capsid protein (P) and Membrane protein (M). The E protein is essential for viral attachment and entry in target cells and contains most of the antigenic determinants of the virus. Protein M is synthesized as the precursor (prM) and serves as a chaperone during maturation of the viral particle (Kuhn et al., 2002). A nucleo-capsid of 30 nm and icosahedral symmetry is surrounded by the lipid envelope. It is composed of the viral genome and capsid proteins. The capsid protein (C) is a highly basic protein with affinity to RNA, and is associated to the genome. Genome is a single stranded positive sense RNA of 11 kb size and consists of 10 genes; three structural and seven nonstructural.

Type I cap structure (m7GpppAmpN2) is located at its 5´-end and the poly(A) tail is absent at its 3´-end. This genomic RNA is translated into a poly-protein by cellular and viral proteases. The poly-protein is processed simultaneously and post-translationally and produces ten mature viral proteins. The N-terminal region

encodes the structural proteins C, prM and E, followed by the non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 respectively. Most of the non-structural proteins are involved in virus replication, which occurs in close association with internal cellular membranes. NS3 and NS5 proteins are multifunctional and multidomain. NS3 has protease, helicase and nucleoside triphosphatase activity. Its function is regulated by its association with other viral proteins. NS5 protein has RNA-dependent RNA-polymerase and methyl-transferase activity. The small non-structural proteins along with NS1 may function for anchoring of the viral replication complex to endoplasmic reticulum (ER) membranes.

TARGET CELLS AND RECEPTOR MOLECULES

Multiple cell types including dendritic cells and monocytes/ macrophages and hepatocytes are involved in Dengue virus infection and replication. Initial recognition and attachment of Dengue virus employs a range of receptor molecules on various target cells. The first group includes carbohydrate molecules such as Sulphated Gylcosaminoglycans (GAGs) and Glycosphingolipids. For example, Heparan Sulphate enhances the virus adsorption to the host cell. The second group includes Lectins (Carbohydrate binding proteins) e.g. DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non integrin). DC-SIGN is expressed on dendritic cells and macrophages under the skin. These are the sites of introduction of Dengue virus by mosquito bite so DC-SIGN serves as first primary target receptor molecule

Another Lectin, Mannose receptor has been reported to contribute viral entry into macrophages. The third group includes Heat Shock Proteins (HSPs) and chaperones which are related to protein folding and it has been reported that Serotype 2 of Dengue virus interacts with these HSPs and chaperon molecules. Other receptor molecules include high affinity Laminin receptor CD14-associated protein and some other uncharacterized proteins. Studies show that Dengue virus uses specific combination of receptor molecules to enter different types of cells and some receptors are commonly employed by different serotypes while others interact specifically with certain serotypes.

LABORATORY DIAGNOSIS

Since there is no specific treatment of Dengue and the management of the disease is symptomatic, early diagnosis becomes even more important. Early diagnosis in not only critical to manage the disease, it is also important in taking effective preventive actions to control Dengue outbreaks. With early diagnosis of Dengue, platelet count can be monitored every day and fluid therapy can be started to avoid shock. Many diagnostic methods are available to support patient management and disease control. The diagnostic method can be chosen according to (i) the purpose for testing e.g. clinical diagnosis, epidemiological survey or vaccine development, (ii) available laboratory facilities (technical expertise, equipments), and (iii) the time of sample collection. Dengue virus, Dengue viral antigens and anti-Dengue virus antibodies appear at different time in patient's blood

and the duration of their persistence is also different.

SEROLOGICAL DIAGNOSIS

Paired serum samples should be collected from the patient at 10 days interval ideally. The first sample should be drawn at the time of onset of illness or as soon as possible. However the convalescent sera can be collected at the time of discharge from the hospital.

Haemagglutination-Inhibition (HI) - HI is sensitive, easy to perform and has been the most frequently used test for Dengue detection. It requires minimal equipment. Dengue virus can agglutinate goose red blood cells under controlled conditions of pH and temperature. The presence of specific HI antibodies can inhibit this process and the potency of this inhibition is measured in an HI assay. To ensure accuracy of the assay other non-specific inhibitors and agglutinins must be removed from serum specimens prior to the test. The assay does not discriminate between infections by closely related viruses e.g. between Dengue virus and Japanese encephalitis virus or West Nile virus. Lack of specificity due to high cross reactivity is the major disadvantage of this test.

Complement Fixation (CF) – The principle of the test is based on the fact that the complement is consumed during antigen-antibody reaction. This test is not routinely used in laboratories due to difficult protocol and the requirement of high technical expertise.

Neutralization test (NT) – In susceptible cell cultures, plaques are formed due to cytopathic effects (CPE) caused by the Dengue virus. Specific antibodies neutralize the virus and prevent the formation of plaques (Plaque Reduction Neutralization Test). This test is the most specific and sensitive test for Dengue. This assay is expensive, time consuming and requires high technical expertise to perform. So, it is not used as routine diagnostic test for Dengue infection.

CONCLUSION

Dengue is caused by Dengue virus which is enveloped, single stranded, positive sense RNA virus of Flaviviridae family. There are four distinct but antigenic ally related serotypes of Dengue virus, Serotype 1, 2, 3 and 4 which are known to infect humans and cause the disease. Infection with one of the serotypes stimulates the production of neutralizing antibodies directed primarily against the envelope protein, conferring lifelong immunity to the serotype. Secondary infection with a different serotype greatly increases the risk of severe Dengue. It is crucial to determine which serotypes of Dengue virus are circulating where and when. Serotyping is routinely being done all over the world and in many parts of our country. Very few studies have been conducted to investigate the trend of circulating serotypes in Rajasthan. This study was therefore planned to standardize a nucleic acid amplification-based test targeting CprM region of the viral genome for the serotyping of Dengue virus and to provide the information of prevalent Dengue virus serotypes in Rajasthan especially in Jaipur and nearby areas. It will provide a ground for further researches on Dengue such as genotyping and antiviral activity of drugs.

Patients with dengue like illness; having pyrexia of unknown origin along with one of any signs and symptoms such as nausea or vomiting, rash, arthralgia or retro-orbital pain were included in the study. Samples were received in the laboratory from different outbreak sites all over the state or were self-collected from patients visiting medicine outdoor unit/ admitted to medical units, SMS and attached group of hospitals.

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