Dengue Fever in Pakistan: Current Updates

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Abstract

Dengue virus (DNV) is the cause of dengue infections and Pakistan along with the world is now being overloaded with their patients. The molecular epidemiological studies reveal that there are four serotypes of the DNV circulating throughout the endemic countries. Non-structural glycoprotein (NS-1) causes the damage of hepatocytes along with the activation of the complement system which in turn causes the severity of the disease. The different modified forms of the PCR have been using for the detection and confirmation of the DNV which includes; Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), Real-time PCR, Nested RT-PCR and Reverse transcription-loop-mediated isothermal amplification (RT-LAMP).

Key Words: Dengue Shock syndrome, DHF, Fatal Disease

Introduction

Infectious diseases are a major cause of mortality in the world and among them now dengue is considered the main human arbovirus. Dengue infection is spread by the bite of mosquitoes and the most common mosquito is Aedes aegypti. This species is found in tropic, subtropic and the Caribbean regions, and usually found with high numbers during the rainy seasons in these areas. Thus disease is more widespread during the rainy season. There is more than 70% disease burden on Asia-Pacific countries.

Dengue Fever (DF) is characterized by headache, retro-orbital pain, myalgia, arthralgia, rash, and in some cases, hemorrhagic manifestations while Dengue Hemorrhagic Fever (DHF) is characterized by the hemorrhagic signs, thrombocytopenia and hemoconcentration or other evidence of vascular leakage which may lead to the Dengue Shock Syndrome (DSS)

Dengue fever is caused by one of the four dengue viruses as DEN-1, DEN-2, DEN-3, and DEN-4. These viruses are different but related. It is complicated to diagnose dengue viruses
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serologically due to the existence of cross-reactive antigenic determinants shared by all four dengue virus serotypes and members of the flavivirus family.

After only a single exposure to a related flavivirus, convalescent patient sera usually contain detectable cross-reactive antibodies\(^2\). In the literature many reports have shown the differences in clinical manifestations among the different dengue serotypes. But the serotypes proven by the antibody detection may have a discrepancy with the real pathogen\(^3,4\).

Kriengsak Limkittikul et al., (2005)\(^5\) found that the white blood cell counts for the DEN1 were the lowest of the 4 serotypes. It was the first publication that proves the comparison on the basis of the differences in hematological responses of the serotypes after dengue infection by the PCR.

**Dengue in Pakistan:** It is an authentic estimation that today about 2.5 5 billion (2/5 of the world population) are the victims of dengue and 50 million are being infected worldwide annually. The mortality rate for those who receive treatment is ranging from1 percent to 2.5 percent. The percentage of death of untreated hemorrhagic dengue is 40 to 50 percent of cases\(^6\).

The first outbreak of DF was reported in 1994 in Pakistan. Then further outbreak occurred in the upper parts of Punjab during 2003. During this era sporadic cases also discovered at Rawalpindi, Islamabad, Peshawar, Jhelum, Abbottabad, Mangla and Haripur\(^7\). During 2006 a largest outbreak has occurred in Karachi which caused the maximum mortality\(^8\). This high mortality rate was appeared due to the co-circulation of DEN-2 and DEN-3 genotypes\(^9\).

Afterwards 2006, Pakistan encountered a significant Dengue problem. Now dengue virus has spread almost all over the country due to elevated number of infected people among the Pakistani population\(^10\).

The high peak level of dengue infection occurs from August to December. Studies of dengue virus infections in Pakistan, Bangladesh and India have shown that it is predominant in males\(^11,12,13\). The present study of clinically proven dengue infections reveals that there is twice number of males than females. This lower rate of infectious females might be due to illiteracy and minimum exposure to this vector borne viral disease. The higher rate is among the young adults between 13-35 years. It is quite similar to the local studies in the karachi\(^11,14\) as well as studies from other endemic regions\(^15,16\).

In September 18, 2011, in the Punjab over 6,000 cases of dengue infections were seen while out of which 5,715 belong to Lahore alone. The Death rate of dengue victims in Pakistan was just 0.04 percent\(^17\).

In Pakistan a report by Jamil B at al., (2007)\(^18\) point out that high prevalence of dengue serotype 3 at Aga Khan University Hospital by analyzing the samples of 39 admitted patients who were confirmed for the presence of IgM anti-dengue by ELISA and PCR\(^18\).
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Serotypes and Genotypes of Dengue Virus:

**Dengue Serotype 1:** The serotype 1 has the following genotypes yet detected\(^{19,20}\). Genotype I (Asia), genotype IV (South Pacific) and genotype V (America-Africa). Each genotype had a well-defined area of distribution, with genotype V (America-Africa) showing the largest geographic expansion. Thirty five DENV-1 strains have been detected in Central and South America and all of them are clustered within genotype V.

**Dengue Serotype 2:** There are four genotypes of DENV-2. Thirty nine DENV-2 strains have been detected and are joined in four different genotypes. These are currently of main epidemiological interest. These are American-Asian, Cosmopolitan, Asian I, and Asian II genotypes.

**Dengue Serotype 3:** There are three genotypes of serotype 3 have been detected as genotype I, genotype II, and genotype III\(^{21}\).

**Dengue Serotype 4:** Five different genotypes of serotype have been detected as genotype I, genotype II, genotype III, genotype sylvatic, and a not previously reported genotype IV.

**Dual infections:** The first case of a dual infection with 2 dengue virus serotypes was reported in 1982 in Puerto Rico in which DEN-1 and DEN-4 circulated in the population during that time was detected and both virus serotypes were isolated from a single patient with DF\(^{22}\). DEN-1 and DEN-3 viruses were isolated from 6 patients with DF in 1989 in New Caledonia\(^{23}\). DEN-1 and DEN-2 were identified In Thailand by RT-PCR in the serum samples of 2 patients with DHF in 1990\(^{24}\). During epidemics in China from 1991 to 1995 eight cases with dual infections of DEN-2 and DEN-4 viruses were identified by RT-PCR in the serum samples of DF patients\(^{25}\). DEN-2 and DEN-3 were isolated from one of 13 cases that were virologically confirmed in Somalia during 1993\(^{26}\).

**Molecular epidemiology:** The global epidemiology of dengue has changed as result in increased frequency of epidemic dengue and occurrence of DHF in the American and Pacific tropical areas along with the occurrence of hyperendemicity in these regions\(^{26,27}\). Modern diagnostic technologies provide powerful tools to diagnose concurrent infections of DNV, and thus now it is possible to determine the frequency of multiple infections with two or more dengue serotypes. It is also possible to check whether concurrent infection is associated with more severe disease or not.

As co-circulation of multiple virus serotypes increases the opportunities for dual infections of humans also increases due to the feeding behavior of Aedes aegypti. This species of mosquito feeds multiple times during a single gonotrophic cycle\(^{28}\). when these mosquitoes were infected experimentally with dengue viruses then it was noted that they spend more time in probing to acquire a blood meal compared with uninfected mosquitoes\(^{29}\) such longer feeding period enhances the host defensive behavior against blood-seeking mosquitoes but increases the possibility that mosquitoes will feed on more hosts to complete their blood meals. This type of feeding behavior increases the chances that they will become dually infected and then subsequently will transmit multiple viruses to a single host.
In vitro experiments, it is demonstrated that infection of human dendritic cells and monocytes results in the increased viral replication due to the suppression of interferon system\textsuperscript{30}. In peripheral blood mononuclear cells taken from the patients with severe dengue fever shown that type 1 interferon associated genes are less abundantly activated compared with milder disease\textsuperscript{31}.

As infection progresses the number of infected cells increases which represent targets for the CD4+ and CD8+ T cells, which then results in large production of interleukin (IL)-10, IL-2, interferon (IFN)-γ and TNF. These singly or in combination might contribute to damage of endothelial cells and alteration of homeostasis. After the release of the produced virions from the infected cells these might also attack on the endothelial cells.

The uptake of non-structural proteins NS1 of the viruses by the hepatocytes might also promote viral infection of the liver\textsuperscript{32,33}. Other factors which correlate with the severity of the illness are the products of complement cascade are C3a and C5a\textsuperscript{34}. NS1 either it is preset in the soluble or membrane-associated form has been demonstrated to activate human complement system. The plasma NS1 and the levels of the terminal SC5b–9 complement complex correlated with the severity of the disease, complement activation and the development of DHF/DSS\textsuperscript{35}.

The pathogenesis of dengue is also explained by the alternative hypotheses which suggests that secondary T-cell responses are blunted because the stimulation of T-cell memory results in the heterotypic CD4+ and CD8+ cells. These cells have little capacity to kill but they secrete inflammatory cytokines that enhance the disease severity\textsuperscript{36}.

Severe form of disease occurs due to dengue viruses of increased virulence. The cross-reactivity between the NS1 and human platelets and endothelial cells causes the increase in number of antibodies that then damage the cells\textsuperscript{37}.

**Tests:** The diagnostic tests which are commonly performed are unable to confirm the dengue infections during the acute febrile phase at a reasonable cost and in a timely manner\textsuperscript{38}. Virus isolation is a time-consuming and fastidious process which requires experienced personnel and specialized laboratory equipments. The development of reverse transcriptase polymerase chain reaction (RT-PCR) and recently real time RT-PCR techniques have significantly reduced the consumption of time and allowed the detection of the virus in the early stage of the infection\textsuperscript{39}. However, these methods remain expensive and technically difficult, particularly in laboratory settings of the developing world.

Serological diagnosis of dengue infection has many advantages including more flexibility, wide availability of reagents, lower cost, and less equipments requirement. while the disadvantages includes cross-reactivity between flaviviruses, half-life of antibody, requirement of paired sera and inability to detect in acute-phase of infection. To over come such disadvantages a number of promising methods have been explored and one of these is the detection of non-structural protein 1 (NS1). It is produced in both membrane-associated and secreted forms and play important role in DNV replication. In infected individuals the amount of secreted form of NS1 is directly correlated with the viremia of infection\textsuperscript{39}. 

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The dengue infections are probably checked by the following tests:

- Elevated IgG titre (that is, 1,280 or greater by haemagglutination inhibition test).
- Fourfold or greater increase in serum IgG (by hemagglutination inhibition test) or increase in IgM antibody specific to dengue virus.
- Detection of dengue virus or antigen in tissue, serum or cerebrospinal fluid by immunohistochemistry, immunofluorescence or enzyme-linked immunosorbent assay (ELISA).
- Detection of dengue virus genomic sequences by reverse transcription polymerase chain reaction (RT-PCR).

**Polymerase Chain Reaction (RT-PCR):** Several PCR based methods for detecting DENV nucleic acid in the serum have been reported in the last decade. These assays have targeted the 3'UTR\(^{40}\), NS5\(^{41,42}\), core\(^{43}\) and the envelope gene sequences.

DF can be diagnosed and confirmed by the process of virus isolation but this procedure is time consuming and generally requires a week for the incubation. Now the most common method being used by the diagnostic laboratories is enzyme-linked immunosorbent assay (ELISA) for IgM antibody produced against dengue virus, or the detection of a rise in IgG antibody titer in the sera.

But the use of IgG antibodies are of little use practically in early management of infected persons. Also IgM antibodies are often absent in the acute phase and may be totally absent in secondary dengue virus infection. In the case of other arboviral infections, IgM antibodies may cross-react with them e.g., yellow fever or Japanese encephalitis and give false results. So, there's a need of rapid laboratory diagnostic tools that help for early and accurate diagnosis and treatment.

The development of PCR helps in the introduction of a number of rapid diagnostic assays for detection of many viruses including DNV\(^{44}\). Different PCR designed experiments have been conducted for the detection of dengue viral RNA from cell cultures of positive human samples and mosquitoes. It limits the time requirements for the results. It permits the serotyping for molecular epidemiological studies and construction of phylogenetic trees to monitor the evolution of geographic strains of dengue virus\(^{45}\). However, such tests are not readily available outside a research center and are not routinely used in the hospitals for the clinical management of patients.

Reverse transcription (RT)-PCR, nested PCR, nucleic acid sequence-based amplification (NASBA), and real-time PCR are the molecular techniques which are based on genomic sequence detection. These are of significant importance for the rapid diagnosis and identification of dengue virus serotypes. In the acute phase serum samples these techniques have been accepted as new standards over virus isolation for detection of dengue.

**Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR):** RT-PCR has been developed for the diagnosis of several infectious diseases and in recent years also being used for the viral infections. It is a specific, sensitive, rapid and can be used for the genome based detection in
human clinical samples, biopsies, autopsy tissues, or mosquitoes. The concurrent infections for the first time were detected by the use of serotype specific monoclonal antibodies and the reverse transcriptase–polymerase chain reaction (RT-PCR).

This molecular test is useful for the diagnosis of dengue infection in the early phase even less than 5 days within infection. It is proven that this test has a sensitivity of 100% in the first 5 days of disease, but reduced to about 70% by 6th day, following the disappearance of the viremia. It is a single step assay which allows the virus to amplify in approximately 1.5 hours. Then these amplified targets are detected by fluorescent probes which replace the need of post amplification electrophoresis.

There are different types of real time RT-PCR that have been developed. These are either singleplex detecting only the single serotype per reaction or multiplex identifying all four serotypes from a single batch of sample.

RT-PCR amplification protocols with the use of dengue oligonucleotide primers can also detect dengue virus RNA in tissue from fatal cases. PCR with specific primers can be used to distinguish among the dengue virus serotypes. PCR with nucleotide sequencing can characterize dengue strains and genotypes. But these genome based assays are costly, demand meticulous technique, and are highly prone to false-positives through contamination, so they are not yet applicable for wide use in all settings. Due to these limitations, the use of RT-PCR is only be considered for the patients who present with diagnostic challenges in the early phase of illness.

In Pakistan during the dengue endemic in 2008, three out of four serotypes (DENV-2, DENV-3 and DENV-4) were identified among the affected persons by this technique.

Real-time PCR: The most widely used test is the nested RT-PCR developed by Lanciotti et al., (1992) and a modification of the same method to single tube format by Harris et al., (1998). More recently real-time PCR based methods have been reported for detection and serotyping of DENV which use fluorescent based reporter chemistries.

It has many advantages over conventional RT-PCR methods due to rapidity. It is nucleic acid-based assays which replace the virus isolation and conventional RT-PCR for diagnosis. But due to high cost of instruments these assays restricted their use to laboratories with good financial resources.

The earlier reports were based on TaqMan probes used multiple probe primer sets for detection of all four serotypes of DENV, while in recently reports were written on DENV group specific real-time PCR used SYBR green based method. KR Gurukumar, et al., (2009) used the TaqMan based real-time PCR for the detection and quantitation of all four serotypes using a single probe primer set targeted against the 3'UTR of DENV. This assay makes it a potential tool for detecting DENV in field-caught mosquitoes.

Majority of the recent reports describe development of the serotype specific real-time PCR for dengue using TaqMan probes or FRET probes. By using these methods serotyping of DENV is done which may not be cost effective for the routine diagnosis because only a small
percentage of samples are positive for DENV RNA during the non endemic season and during the active transmission season only about 50% of samples may be positive for DENV RNA.

For the initial screening of the infected samples a group-specific PCR would be a useful tool and only the samples with positive result for DENV then can be subjected to serotyping. Lai et al., (2007) Initially screened samples by a SYBR green based group specific real-time PCR and then positive samples were serotyped by a duplex or a fourplex TaqMan based assay so thereby reduced the operation cost on diagnosis by half. However SYBR green-based detection systems has a major disadvantage of false positives results due to the binding of dye to the primer dimers or to non specifically amplified DNA. Then other analyses such as Melt curves are often used to confirm the fidelity of the reaction. Mostly group specific real time PCR assays reported are also SYBR green based.

Callahan et al. first described The DENV group specific assay using TaqMan probes. The fluorescent probes in TaqMan assay are known to be target specific and sensitive to mismatches. All four serotypes of DENV from clinical samples or cell cultures or infected mouse brain or mosquitoes could be detected by the qRT-PCR. There is no amplification chance with the related Flaviviruses or with samples from other febrile illness.

This test has the highest sensitivity for DENV-2 in terms of PFU as 0.001 PFU followed by DENV-1 and DENV-3 at 0.01 PFU and DENV-4 had the lowest sensitivity of 1 PFU. The reason for the difference in sensitivities could have been due to a difference in the proportion of non-infectious RNA transcripts to infectious particles.

**Nested RT-PCR:** The nested RT-PCR was developed by Lanciotti et al., (1992). It is a two-step technique which is now being used worldwide and in most of the laboratories for virus detection. The identification and typing of dengue virus serotypes is done by RT-PCR, followed by nested PCR with complex and serotype specific primers, respectively. This method was then later modified to a single-step multiplex RT-PCR system for the detection and typing of dengue viruses.

These such existing RT-PCR test systems are less sensitive and the assays done by them are time-consuming (3 to 4 h) and much more complicated, with several steps of amplification (cDNA-PCR-nested PCR) that require the use of a high-precision thermal cycler. For precise detection there is a need of more sensitive and real-time-based assays many investigators have emphasized and reported on fully automatic nested RT-PCR assays for the accurate detection of dengue virus serotype in acute-phase serum samples.

**Reverse transcription-loop-mediated isothermal amplification (RT-LAMP):** This assay is very simple and fast because the amplification can be obtained within the thirty minutes under the isothermal conditions at 63°C. This is done by employing a set of four serotype specific primer mixtures. This method targets the 3’ coding region for detection of viral serotype. This is advantageous method due to its simple operation, rapid reaction, and easy detection.

In this assay, the reaction is carried out in a single tube by mixing of the buffer, primers, reverse transcriptase, and DNA polymerase. Incubation of the mixture is done at 63°C for 1 h.
comparison with the RT-PCR and real-time PCR it has the advantages of reaction simplicity and detection sensitivity. Under isothermal conditions the reactions goes on by employing six primers that recognize eight distinct regions of the target. It produces large amount of a by-product which is pyrophosphate ion. It leads to the white precipitation of magnesium pyrophosphate which causes the turbidity. Increase in turbidity correlates with the amount of DNA synthesized. So monitoring of the reaction mixture can be achieved by the measurement of turbidity. It can also be useful in the quantitation of the virus load in a clinical sample, which helps in the understanding of the viremic status of the patient. It also shows higher sensitivity by correctly picking samples with low levels of virus that might missed by RT-PCR and nested PCR. It shows no false positive results with any other of the serologically related flaviviruses tested or with serum samples from healthy humans, indicating that it is highly specific for the target sequence.

**Advantages:** There are the following advantages of using PCR for the dengue virus serotype detection.

- ability to determine dengue serotypes,
- rapid so less time-consuming,
- quantitative measurements are easily obtained,
- a lower contamination rate,
- a higher sensitivity,
- a higher specificity,
- easy standardization,
- Detection in early stage of dengue infection. Before the severity of the diseases virus can be detected.

It is now being widely used for the diagnosis of dengue instead of virus isolation due to these advantages.

**Limitations:** There are following disadvantages of using PCR for dengue virus detection;

- availability only in a few centers with facilities and experienced lab workers,
- very expensive,
- requirement of special storage temperatures,
- short transportation time between collection and extraction,
- Usefulness in early phase of illness (less than 5 days).
**Future Prospects:** Dengue infections are now current endemics in Pakistan. To limit the occurrence there must be fast and accurate diagnostic tools for the detection and confirmation of the dengue virus within the body. PCR tests due to their limitations are rather being efficient method for detection and it must be further evaluate to overcome this problem. Along with other expenditures on the prevention of dengue infection there must be the little bit increase of use of such PCR experiments on dengue virus to understand the molecular epidemiology and seroprevalence of the dengue in our beloved country. To whom who want to work for the diagnosis of DNV this paper would be very helpful for him/her.

**References**


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