Clinicoepidemiological study of dengue positive patients admitted to tertiary care hospitals in the Burdwan district, using molecular seroprofiling and enzyme linked immunosorbent assays of cytokines and chemokines

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Background: The World Health Organization views dengue fever as a significant worldwide public health threat in the tropics and subtropics. Dengue virus has four serotypes, DENG 1 to 4, which were responsible for outbreaks that occurred in other parts of the world from the 1980s until now. Aims and Objectives: Clinical manifestations and molecular and serological studies help determine the inflammatory response events associated with dengue severity. This study aimed to define the profiles of some cytokines and chemokines, as well as their molecular serotyping. Materials and Methods: Blood serum samples are collected, followed by serodiagnosis (NS1 and IgM) through ELISA, molecular serotyping detection of dengue by real-time PCR through SyBr green, and an immunological study of cytokines tumor necrosis factor-alpha and interferon-gamma (IFN-γ) on mother-hand chemokines CCL26 and MCP1 by ELISA. Statistical analysis was performed by SPSS (version 22). Results: The study examined 70 dengue seropositive samples, of which five were NS1 positive and 55 were IgM positive. After accounting for comorbidities and patients' demographic classifications, the 21–30 age group has a maximum positivity of 30%. The mean concentration level of IFN-γ is elevated at 1544.438 pg/mL. Conclusion: Dengue-positive patients have a high ischemic heart disease rate, among other diseases. An elevated circulating level of osteopetrosis may cause cancer, tuberculosis, and other serious diseases. In immunological aspects, IFN-γ elevation can be used to predict the severity of dengue infection.

Key words: Dengue; ELISA; Real-time-PCR; Serotype; DENG-3; Interferon-gamma; Cytokines and Chemokines; Co-morbidites

INTRODUCTION

The dengue virus is an arthropod-borne virus that belongs to the family Flaviviridae and has four distinct serotypes (DEN-1, DEN-2, DEN-3, and DEN-4).¹ The World Health Organization (WHO) views dengue fever (DF) as a significant worldwide public health threat in the tropics and subtropics. Between 1960 and 2010, dengue cases rose by 30 times globally as a result of rapid population expansion, climate change, unplanned urbanization, ineffective mosquito control, and global travel.
control, frequent air travel, and a dearth of medical services. There are 2.5 billion individuals in dengue-endemic areas, 400 million illnesses occur annually, and the fatality rate can exceed 20% in some places. The first instance of dengue-like sickness to be documented in India happened in Madras in 1780, and the first epidemic of DF to be virologically proven in India took place in Calcutta and the Eastern Coast of India in 1963–1964. All four dengue virus serotypes were responsible for significant outbreaks that occurred in other parts of the world throughout the 1980s and 1990s.

Clinical manifestations of dengue virus infection are varied, ranging from asymptomatic sickness through DF to the severe condition known as dengue hemorrhagic fever or dengue shock syndrome (DHF or DSS). DF (DHF) has been linked to oral mucosal damage DF since the 1950s, although oral characteristics are more typically linked to DF than to DHF. The first pandemic broke out in Manila in 1954 and other nations began to experience this gradually. The global development of dengue infections may be explained by the fact that the more virulent genotypes of the virus are replacing the less virulent ones. The E protein is organized on the surface of DENVs as 90 closely packed monomers that lay flat against the host cytoplasm. The E protein enhances viral entrance into host cells by binding to cellular receptors and facilitating the fusion of viral and cellular membranes. New DENV particles can either include processed M protein on release from infected cells, making them infectious or “mature.” The etiology of severe illness has been linked to non-structural proteins (NS1–NS5) produced in membrane-associated and secreted forms. NS1 is expressed on the surface of infected cells rather than being a component of the virion. When compared to patients with DF, patients with DHF had greater plasma levels of secreted NS1. Furthermore, individuals who are at risk of developing DHF are identified by having higher free NS1 levels within 72 h after the commencement of the illness. Acute-phase samples from individuals with secondary dengue infections but not initial infections show very high levels of the NS1 protein. This shows that NS1, which is known to play a significant role in the pathogenesis of severe dengue infections, may help to create circulating immune complexes. The Japanese encephalitis virus and the dengue virus both share antigenic epitopes. An important role in the spread of dengue is played by mosquitoes of the genus *Aedes aegypti*, the main and most significant vector; however, depending on the region, *Aedes albopictus* and *Aedes polynesiensis* may also function as vectors. For example, it has been discovered that *A. albopictus* can occasionally transmit dengue in Thailand, Samui Island, India, Singapore, and Mexico.

To better understand the inflammatory response events associated with dengue severity, this study aimed to define the profiles of some cytokines and chemokines along with molecular serotyping. This might help identify biomarkers for the proper triage of patients with respect to infected serotypes.

**Aims and objectives**

To identify different serotypes of dengue virus and to evaluate the cytokines and chemokines concentration level in dengue positive patients.

**MATERIALS AND METHODS**

This institutionally based cross-sectional study was conducted at the Department of Microbiology, Burdwan Medical College, West Bengal, during the tenure of September to November 2022 (3 months). A total of 70 patient samples were collected from dengue IgM and NS1 seropositive patients. Samples were submitted with properly completed consent forms and clinical manifestations documented.

**Ethical statement**

This cross-sectional study has been approved by the Institutional Ethics Committee of Burdwan Medical College and Hospital.

**Collection of samples**

The blood samples were collected in a Clot Vial (red) after 30 min at the room temperature centrifuged the sample 1000 g in 30 s and separate the serum for further processing.

**Serodiagnosis (NS1/IgM) through ELISA**

All the patients’ serum samples were collected and serodiagnosis of IgM and NS1 is done using STANDARD E Dengue IgM Capture ELISA manufactured by standard deviation (SD) Biosensor Healthcare Pvt. Ltd. for IgM testing and Oscar Medicare Pvt. Ltd. used for NS1 testing, as per manufactures instructions. Among all the IgM/NS1 dengue positive samples, 70 samples were selected based on their clinical manifestations.

**Viral RNA isolation**

The patient’s RNA from the serum sample was isolated using the HiPurA Viral Automated RNA Purification Kit (HiMedia). RNA concentration was measured by measuring OD values using a spectrophotometer.

**C-DNA synthesis**

The extracted patient’s RNA samples then convert into C-DNA by Prime Script™ 1st strand cDNA Synthesis Kit (HiMedia) as per the manufacturer’s protocol.

**Oligonucleotide design and synthesis**

Dengue virus nucleotide sequences were obtained from GenBank. Primers were generated and tested for
quantitative real-time PCR (RT-PCR) after identifying potential target areas. Table 1 shows the final sequences of specific primer pairs.

**Molecular detection of H1N1 by RT-PCR**

The SyBr Green qualitative RT-PCR reactions that serotyped dengue (DENG1–DENG4) were performed using TB Green Premix Ex Taq II (Tli RNase H Plus), manufactured by Takara Bio. Each reaction contained 1 TB of Green Premix master mix and primers designed for a short sequence of polyprotein genes from different dengue serotypes, namely, DENG1, DENG2, DENG3, and DENG4. Bio-Rad CFX 96 RT-PCR conditions consisted of an initial denaturation incubation at 95°C for 2 min, followed by 38 cycles of alternating 95°C incubations for 5 s, 56°C incubations for 30 s, and 72°C incubations for 10 s. Fluorescence was detected after every 72°C extension incubation. This method was used to screen 70 clinical samples, allowing us to confirm the serotypes of dengue virus-infected individuals in the Burdwan district. The presence of the housekeeping gene Beta Actin was used as an internal test control to assess each specimen’s DNA suitability for PCR amplification.

**Immunological study of cytokines and chemokines by ELISA**

The short-term plasmatic leakage caused by the host immune response is thought to be the crucial point in dengue physiopathology. Soluble immune mediators in the blood have been linked to severe illness outcomes such as shock and haemorrhages. Among these are inflammatory cytokines such as tumor necrosis factor (TNF)-alpha and interferon-gamma (IFN-γ) and chemokines such as MCP-1, which have all been found in dengue patients. We chose those two cytokines and two chemokines for this study to assess their concentration levels in dengue positive patients. Blood samples from 70 dengue positive patients were collected and stored at −80°C. Cytokines and chemokines were quantified through specific ELISA kit sets manufactured by Krishgen Biosystems, and the test was performed with the help of the manufacturer’s instructions. This study measured TNF-alpha and IFN-γ under the cytokines group, and CCL26 (Eotaxin-3) and MCP-1 concentrations were measured under the chemokines group. All tests were performed, including standards, which were measured as per the manufacturer’s guidelines, and OD values were measured by an ELISA reader (manufactured by J. Mitra). Figure 1 depicts the overall workflow of the project.

**Statistical analysis**

Analysis of all the data was performed by SPSS software (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). Qualitative variables were expressed as mean±SD.

**RESULTS**

**Patients clinical and demographic characterization**

Among 70 dengue positive patients, the dominant age group is between 21 and 30 years and their positivity rate is 30 %. In Table 2, different age groups with their seroprevalence with positivity rate were described. In this study, we found that 1.43% of positivity rate in the above 61 years of age group which is lower than others. Among 70 patients, 50 were male patients and 20 female patients positive. In the sight of seroprevalence NS1 shows 5 positive, IgM shows 55 positive and combined showed 10 individuals positive.

In this study, among 70 dengue positive patients different comorbidities found. Diabetes mellitus (35.71%), hypertension (44.29%), ischemic heart disease (IHD) (54.29%), bronchial asthma (17.14%), chronic liver disease (15.71%), pulmonary tuberculosis (14.29%) found in those 70 dengue positive patients (Table 3).

In our total study time, we selected those 70 seropositive patients for molecular serotyping through RT-PCR of dengue virus circulating in Burdwan district, West Bengal. DENG3 (97.14%) is dominant with strong coinfection with DENG2 (82.86). DENG4 (5.71%) was found lowest infection (Table 4). DENG1 shows also mixed infection in some patients. In Supplementary file, the molecular serotyping with CT values is described.

<p>| Table 1: Degenerated primer sequences for the dengue group- and serotype-specific one-step SYBR Green-based RT-PCR assay |</p>
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon (bp)</th>
<th>Primer Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENG1</td>
<td>D1F</td>
<td>TCAATATGCTGAAACCGCCGAGAAACCG 482 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG1</td>
<td>DS1</td>
<td>TGTTGTCTTAAACAAAGGAGAGGTC 389 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG2</td>
<td>D1F</td>
<td>TCAATATGCTGAAACCGCCGAGAAACCG 482 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG2</td>
<td>DS2</td>
<td>CGCCACAAGGGGCGATGACAG 119 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG3</td>
<td>D1F</td>
<td>TCAATATGCTGAAACCGCCGAGAAACCG 482 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG3</td>
<td>DS3</td>
<td>TAAACATCATCATGAGACAGAGAGGTC 290 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG4</td>
<td>D1F</td>
<td>TCAATATGCTGAAACCGCCGAGAAACCG 482 10uM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DENG4</td>
<td>DS4</td>
<td>TCAATATGCTGAAACCGCCGAGAAACCG 482 10uM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
After the statistical analysis, the correlation between molecular serotyping DENG1-4 with IgM/NS1 serodiagnosis was described in Table 5 with their P-value. The statistical approach was made 2 sided asymmetrical manner. In this result DENG1 to DENG4, none of them shows a statistically significant because their value much higher than 0.05.

In this study, another statistical analysis was done and developed a correlation with comorbidities with four different dengue serotypes. In Table 6, there is a two-sided
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Crosstab statistical analysis was made and their P-value was given. No values were statistically significant in respect to comorbidities with dengue.

The mean concentration level of IFN-γ was elevated in the 70 patient's serum rather than TNF-α in the cytokines category. 15.44.438 pg/mL was the mean conc. of IFN-γ. In the chemokines category MCP-1, mean conc. was dominant than CCL26 among 70 dengue positive patients. The mean concentration level of MCP1 was 662.248 pg/mL. In total four IFN-γ, concentration was high than others. Cytokines and Chemokines mean concentration with SD were described and illustrated in Table 7 and Figure 2.

**DISCUSSION**

The four main serotypes of the dengue virus (DEN-1, DEN-2, DEN-3, and DEN-4) are arthropod-borne viruses that belong to the genus Flavivirus and family Flaviviridae.1,6 Dengue is regarded by the WHO as a serious global public health threat in tropics and subtropics countries. Due to factors such a faster pace of population expansion, climate change, unplanned urbanization, ineffective mosquito control, frequent air travel, and a lack of medical services, dengue cases increased by 30 times globally between 1960 and 2010.2,6,17 There are 2.5 billion people who live in dengue-endemic area,2 and 400 million illnesses happen year with a fatality rate that exceeds 5–20% in some places.3 More than 100 nations are affected by dengue illness, including Europe and the USA.18 The first instance of dengue-like sickness to be documented in India occurred in Madras in 1780, and the first epidemic of DF to be virologically proven in India took place in Calcutta and the Eastern Coast of India in 1963–1964.4 The clinical picture of dengue virus infection is varied and includes asymptomatic sickness, DF, and the severe illness known as (DHF/DSS).5 Although oral symptoms are more typically linked with DHF than with DF, oral mucosal involvement is present in about 30% of patients.7 Since dengue virus infection has a variety of clinical manifestations, a precise diagnosis is challenging and depends on laboratory confirmation. Since there is now no antiviral medication available, the problem typically resolves on its own.

In this study, DENG3 (97.14%) is predominant in Burdwan district but in among India in 51 research, dengue serotype information was available. The most common serotype in the four investigations from the northeast was DEN-3, which was followed by DEN-1 and DEN-2 serotypes.19 All four dengue serotypes were found to be in circulation according to published studies from India, with DEN-2 and DEN-3 being the most often reported serotypes. More than one serotype was identified to circulate in two-thirds of the studies. An analysis showed that one-fourth of the cases were severe and that more than two-fifths of the laboratory-verified illnesses were secondary infections.20 After statistical analysis, we found no significant relation in between molecular serotyping with serodiagnosis among 70 dengue positive patients. The P-value was much higher than 0.05.

![Figure 2: Concentration Level of different cytokines and chemokines among 70 dengue positive patients](image-url)
An increase in TNF-α and MCP-1 can be used to predict the severity of dengue infections. This may assist in triaging patients as it may be difficult to assess the severity at the time of diagnosis. Early identification of more advanced disease together with more proactive management may result in favorable outcomes. Studies have shown that increased mean concentration level of TNF-alpha among 70 dengue positive patients. IFN-γ is a main cytokine released by CD8 effector T cells, capable of blocking viral replication. IFN-γ is also a potent activator for phagocytic cells, increasing their activity as well as their ability to produce cytokines. IFN-γ has been shown to increase permeability of endothelial cells and to upregulate expression of TNF receptors on myeloid and epithelial cells, which can make them more sensitive to TNF-α exposure. IFN-γ is vital to tumor surveillance by the immune system and a high correlation between IFN production and tumor regression has been seen in immunotherapy. It is anti-angiogenic, inhibits proliferation, sensitizes tumors cells to apoptosis, upregulates MHC class I and II expression, and stimulates antitumor immune activity. There have been mixed results as to the effectiveness of IFN-γ in the clinical treatment of various cancers. IFN-γ activates alveolar macrophages, which are important in host immunity against M. tuberculosis. Co-administration of IFN-β and anti-TB to TB patients led to a reduction in cavitary lesion sizes and a decrease in the mycobacterial burden. IFN-γ has been used in the treatment of scleroderma because of its antifibrotic activity; its ability to reduce collagen production in vitro, and to inhibit fibroblast cell proliferation. In most clinical trials, either subcutaneous or intramuscular administration of the drug has resulted in modest improvements in patients with the disease. In our study, plasma levels of IFN-γ were not related to the presence of ascites, which are considered a marker of plasma leakage. The study’s findings on the putative pathogenetic involvement of IFN-γ in the induction of plasma leakage were inconclusive. Due to this, it is impossible to draw definitive conclusions about how both cytokines contribute to the pathogenesis of dengue virus infection from the data.

**Limitations of the study**
Sequencing should be done to identify any mutation. We have not done it.

**CONCLUSION**
In this study, the clinical picture of dengue cases in Burdwan, along with the cytokines and chemokines responses. In the age group of 21–30 years were dominant and DENG3 prevalence higher than others. Coinfection of DENG2 and DENG3 occurs prominently after molecular serotyping through RT-PCR. IHD is a top most comorbidity found among dengue positive patients in Burdwan district. Higher level of IFN-γ may leads to cancer, tuberculosis, osteopetrosis, scleroderma, etc. which may a leads to serious threat for mankind. IFN-γ may play a prognostic biomarker for progression of such kind of disease after exposure of dengue virus infection. Our results suggested a downregulation of IFN-γ has traditionally been thought of as a pro-inflammatory cytokine that regulates anti-inflammatory responses. Therefore, lowering the pro-inflammatory cytokines could be a preventive measure and is hypothesized to be a possible drug target area.

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Authors' Contributions:
NC- Concept and design of the study, prepare the 1st draft of manuscript, preparation of manuscript; TB- Concept and Design of the study, Prepare the 1st draft of manuscript, Preparation of manuscript; SN- Prepare review of literature and preparation of manuscript; AN- Perform the laboratory test, prepare the result and interpretation; NM- Perform the laboratory test, prepare the result and interpretation; SM- Sample collection and documentation; KD- Sample collection and documentation.

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