Research Article

Comparative analysis of clinicopathological findings with immunological and molecular tests for early diagnosis and confirmation of dengue in a tertiary-care hospital in Gwalior

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Received July 2, 2015. Accepted July 20, 2015

Abstract

Background: Dengue is a potentially life-threatening condition that can occur in people of all age groups. Dengue virus is distributed worldwide and represents a serious public health problem.

Objective: To perform immunological diagnosis and further confirmation by molecular methods in suspected cases of dengue virus infection and compare their results of clinicopathological findings, serology, and molecular techniques for early confirmation and further clinical correlation.

Materials and Methods: The study was done for the patients who were suspected for dengue for a period of 2 years. Routine hematological investigations, including platelets, were done by automated autoanalyzer, and screening for dengue NS1 antigen detection by Panbio rapid strip test and dengue IgM and IgG antibodies by duo cassette Panbio was performed. Dengue virus isolation was done by using QIAamp viral RNA mini kit-RT-polymerase chain reaction (PCR).

Result: Of the total 120 suspected cases for dengue, the maximum incidence in relation to age was noted in the age group of 16–30 years, and the prevalence rate was higher in male patients. We also found that nine (7.5%) cases and 29 (24.17%) cases were positive for NS1 antigen and IgM antibody, respectively. Among the total 120 cases of IgG test by antiflavi card, 10 (8.33%) cases were positive, and the results were compared with those of RT-PCR. Of the total 38 NS1- and IgM-positive cases, we found that a total of four (10.5%) cases were found with RT-PCR positivity.

Conclusion: Involvement of many laboratories in the diagnosis of dengue coupled with general awareness among the public and constant vigilance by the health-care officials could go a long way in combating dengue, especially in the prevention of large outbreaks by monitoring dengue viral activity by serological and molecular tests.

KEY WORDS: Dengue, NS1 antigen, IgG, IgM, RT-PCR

Introduction

Infections caused by viruses are exorbitantly prevalent in the world. The viruses as causative factors of disease have existed in the world from time immemorial and have concurrently inflicted hazardous and lethal diseases to human beings, animals, and plants alike. With respect to humans, in particular, there have been instances of infections caused by extremely dangerous viruses, resulting in immense mortality together with morbidity. During the last few decades of the twentieth century, viral diseases have been emerging and reemerging in large scales with pronounced virulence such as plague, dengue, Japanese encephalitis, viral hepatitis, and a*cquired immune deficiency syndrome*.[1] The majority of these emerging diseases belong to the group of arthropod-borne viral infections because of massive urbanization, crowding, spiraling international travel, changes in lifestyle, and compatible ecosystem resulting in the resurgence and increase in the vector population.[2] These arboviral infections

Access this article online	
Website: http://www.ijmsph.com	Quick Response Code:
DOI: 10.5455/ijmsph.2016.0207201534	

account for nearly 90% of the emerging and reemerging diseases. Of them, dengue is an old disease; recent decades have seen an unprecedented increase in the geographic range, incidence, and severity of the dengue infections.[2]

Dengue is a potentially life-threatening condition that can occur in people of all age groups. It is estimated that there are approximately 20 million cases of dengue infection in the world resulting in around 24,000 deaths annually.[4] Dengue virus is distributed worldwide and represents a serious public health problem in southeast Asia, the Caribbean, the Pacific islands, and Latin America.[1,4] Dengue virus belongs to the genus *Flavivirus* under the family Flaviviridae. There are four closely related but antigenically distinct dengue virus serotypes (DEN-1, DEN-2, DEN-3, and DEN-4). Dengue is transmitted to human population by *Aedes/Stegomyia aegypti* mosquito, which happens to be the natural vector of dengue virus. This mosquito is also a vector of other *Flavivirus* (e.g., yellow fever) and alpha virus (e.g., chikungunya). Dengue viruses maintain a man–mosquito–man cycle, and, therefore, they may disseminate the disease very rapidly in places with a high density of human population. Therefore, mosquito is considered only as an accessory vector of dengue virus.[5] Dengue virus infection ranges from classical dengue fever (DF) to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) with either benign or classical symptoms.[6]

The most challenging aspect of dengue infection is its proper diagnosis. It can be detected by isolating and identifying the dengue serotype involved and by the determination of IgM or IgG antibodies in the patient's sera.[4] All the dengue viral serotypes (DEN 1–4) and other members of the flaviviral family exhibit the cross-reactive antigenic determinants, which make the serodiagnosis complicated. This results in a great number of false positivity, as apart from the flaviviruses, many sera samples of typhoid and malaria also show false-positive results. Hemagglutination inhibition (HI) test is the most widely used and the WHO-recommended diagnostic test. A fourfold or greater increase in antibody titer with paired serum samples is confirmatory for diagnosis. IgM capture enzyme-linked immunosorbent assay (MAC ELISA), Dot ELISA, and immunochromatographic tests have also been extensively evaluated for the detection of dengue virus-specific antibodies. Dot ELISA based on dipstick ELISA principle is a field-based semi-quantitative assay and can be used to determine the level of antibody in both acute and convalescent phases.[7]

A very rapid immuno-based immunochromatic test has also been developed for the diagnosis of dengue. So far, the research on the development of an effective vaccine against dengue is not successful, as there is no treatment for dengue patients. So, patient care is the most important. The early diagnosis of dengue can help in formulating a proper strategy for patient management and treatment. So, on the basis of significance of dengue infection, this study was undertaken to perform immunological diagnosis and further confirmation by molecular methods in suspected cases of dengue virus infection and compare their results of clinicopathological findings, serology, and molecular techniques for early confirmation and further clinical correlation.

Materials and Methods

This prospective study was carried out in the Department of Pathology, Gajra Raja Medical College, Gwalior, Madhya Pradesh, India, in association with Defence Research and Development Establishment (Ministry of Defence, Government of India), Gwalior. Ethical clearances were obtained from Medical Research Ethics Committees of Medical College, Gwalior. All the specimens were received from the patients of JA Group of Hospitals, Gajra Raja Medical College, Gwalior, for a period of 2 years in which the patients were screened for dengue virus infection. Two sets of blood samples were collected in two vials from a total of 120 patients, one containing anticoagulant and another plain vial taking all the aseptic precautions. Routine hematological investigations including platelets were done by automated autoanalyzer, and screening for dengue NS1 antigen detection by Panbio rapid strip test and dengue IgM and IgG antibodies by duo cassette Panbio as per the manufacturer's instructions were performed. Dengue virus isolation was done by using QIAamp viral RNA mini kit (Qiagen, Germany)-RT polymerase chain reaction (RT-PCR) according to the manufacturer's instructions.

Result

In this study, a total of 120 patients were suspected of dengue fever syndrome with complaints of high-grade fever, headache, body ache, and retro-orbital pain. Of these 120 cases, there was no case of clinical DHF. A battery of diagnostic tests was performed to identify the presence of dengue fever-causing viruses by direct and indirect techniques for diagnosis. All these samples were screened for antigen (NS1) detection, dengue-specific IgM and IgG antibodies (for the detection of antibodies), and RT-PCR (for the detection of RNA). The most frequent detection and isolation of the dengue in relation to age was noted in the age group of 16–30 years (52.3%), followed by 31–45 years (23.3%), 0–15 years (22.5%), 46–60 years (11.67%), and > 60 years (7.5%). We also found a relationship between dengue and sex. The prevalence rate was higher in male patients (71.66%) when compared with female patients (28.33%). We also compared the relationship between dengue and hematological parameters of patients. Of the 120 cases, anemia was seen in 23 (19.2%) patients, leukopenia in 36 (30%) patients, lymphocytosis in 86 (71.66%) patients, thrombocytopenia in 68 (43.33%) patients, and decreased value of hematocrit in 48 (40%) patients, which is shown in Table 1.

We found that, of the total 120 cases, nine (7.5%) cases were positive for NS1 antigen. Among them, seven (5.83%) and two (1.67%) cases were male and female patients, respectively. Twenty-nine (24.17%) and 91 (75.8%) cases were positive and negative, respectively, for IgM antibody. While we compared with the age group, we found that a total

Table 1: Hematological parameters of patients

Variables	Cases	%
Hb (g%)		
<10	23	19.2
10–12	68	56.7
>12	29	24.2
TLC (/mm^3) (4,000–11,000)		
Normal	65	54.2
Increased	19	15.8
Decreased	36	30
DLC (%)		
Lymohocitosis	86	71.2
Normal DLC	34	28.3
Neutrophilia	0	0
Platelet count (lac/mm^3) (1.5–4.5)		
Normal	52	43.3
Reduced	68	56.7
Increased	0	0
PCV% male (40%–50%), female (38%–45%) subjects		
Normal	72	60
Increased	0	0
Reduced	48	40

of 27 cases were aged younger than 15 years, and of them, 11 (40.7%) were positive for IgM antibody; a total of 93 cases were of the age group older than 15 years, and of them, 19.35% were positive for IgM antibody. We also compared a total of 29 IgM-positive cases with relation to sex, and we found that a total of 19 (65.5%) and 10 (35.5%) cases were male and female patients, respectively. We also compared combined dengue IgM and IgG-positive cases among the total 29 dengue IgM-positive cases. We found that a total of nine (31.03%) cases were positive for IgG also. We also compared the distribution of the combined dengue IgM and IgG-positive samples in children and adults among IgM-positive cases. We found that of the total 11 IgM-positive children, two (18.8%) cases were combined IgM and IgG positive, and of the total 18 IgM-positive adults, seven (38.8%) were combined IgM and 1gG positive. Among the total 120 cases of IgG test by antiflavi card, 10 (8.33%) and 110 (91.7%) cases were positive and negative for IgG, respectively. We also compared sex-wise distribution of IgG-positive cases; we found that, of the total 10 IgG-positive cases, seven (70%) and three (30%) cases were male and female patients, respectively. We also compared the results of RT-PCR for isolation of dengue virus in the total 38 NS1- and IgM-positive cases and found that a total of four (10.5%) cases showed RT-PCR positivity, and of these four positive cases, three (7.9%) and one (2.6%) were male and female patients, respectively.

Discussion

Dengue is an important emerging disease of the tropical and subtropical regions today. The WHO reported that, worldwide, nearly 2.5 billion people continue to live at-risk of contracting the infection, while 50 million cases and 24,000 deaths are estimated to occur in 100 cpimtroes.[8] In most of the studies, male subjects were found to be more affected than female subjects. There was a study done in New Delhi, which reported the ratio of male and female as 3:1.[9] Another study was done in Udupi, the ratio of male and female was 1.8:1, while another study from Surat during the year 2009 to 2011 reported male and female ratio as 2.54:1.[10] In this study, this ratio was 2.2:1, and our results showed the general trend of male preponderance in incidence, and it was similar to the study done at Surat. The possible reasons of increased incidence of dengue in male subjects may be related to more mobility of male subjects in search of jobs or otherwise when compared with females, and because of this, more chances prevail to contact mosquito-borne dengue infections. During various epidemics in India, in general, children aged younger than 15 years were quite severely affected, but the majority of infections occurred in active adults in the age group between 16 and 60 years.[8] Barde et al.[11] also observed that the disease is more severe in children than in adults.

Monath[12] reported that DHF and DSS are the major causes of hospitalization and mortality, mainly among children, in tropical and subtropical countries. Ong et al.[13] also suggested that, possibly, children are dengue naive and those adults who have been previously infected by dengue virus infection possess some immunity with one or more dengue viruses and experience milder course of disease. Alcon et al.[14] stated that nonstructural protein (NS1) is released from infected mammalian cells but not vector-derived insect cells. Halstead[15] suggested that NS1 antigen test is the inexpensive, rapid, sensitive, and specific test for the diagnosis of dengue during early febrile stage. In this test, dengue group-specific NS1 M antibody in an ELISA format is used to detect dengue NS1 antigen in blood. NS1 test can confirm 85% of PCR-positive samples. Alcon et al.[14] found that NS1 antigen was found circulating from the first day after the onset of fever up to 9 days, once the clinical phase is over. They suggested that NS1 protein detection may allow an early diagnosis of infection. They also found that NS1 circulation levels varied among individuals during the course of the disease ranging from several monograms per milliliter to peak 50 μgm/mL of serum. NS1 concentration did not differ in serum specimens obtained from patients of primary and secondary dengue virus infections. Libraty et al.[16] also found that free NS1 levels in plasma correlated with viremia levels and were higher in patients with DHF than in those with DF. An elevated free NS1 level of 600 ng/mL or more of 72 h of illness onset correlated with patients with a high risk of developing DHF. Vaughn et al.[17] found that dengue infection severity was very well correlated with a high titer of circulating virus in early illness blood sample along with NS1 titer. Libraty et al.[16] also observed that, in acute dengue viral infection, peak viremia provides the best quantitative estimate of cellular infection, but this event is transient and happens early in infection; therefore, dengue NS1 protein production, which parallels cellular dengue infection, is the preferred investigation in early disease. Alcon et al.[14] in a wide-based cohort of dengue virus-infected

patients in French Guiana (from year 1996 to 1997) demonstrated the presence of NS1 in serum of 0–9 days of dengue infection. They found 80% positivity in the first 6 days of infectivity. Between 10 and 66 days, none of the sera showed detectable levels of NS1, presumably owing to the appearance of antibody response to complex viral antigens. Convalescent phase antisera shared dengue virus-specific antibodies. In our study, all the 120 cases of clinically suspected dengue fever were tested for NS1 and a total of nine (7.50%) cases were found positive. The wide variation in the positivity of results of Alcon et al.[14] and our study is possibly caused by the confirmed cases of dengue by serological and molecular tests, while this study was conducted in clinically suspected cases of dengue. Ruechusatsawat et al.[18] stated that, in primary infections, serum IgM antibodies can be detected from dengue patients as early as 3–5 days after the onset of fever. Innis et al.[19] found that secondary infection of dengue is characterized by high IgG levels that may or may not be accompanied by elevated IgM levels. Blacksell et al.[20] reported a high rate of false-positive results using MAC ELISA tests. Halstead[15] reported that a substantial amount of IgM dengue antibodies are lost on adsorption, reducing the diagnostic accuracy and sensitivity; therefore, filter paper-based methods should not be relied for IgM antibodies test for dengue. However, because IgG antibodies are not absorbed to filter paper, such caution is not necessary for the detection of IgG antibodies in secondary dengue infection. Vijayakumar et al.[21] from Vellore studied IgM antibody positivity for five consecutive years in dengue cases, and they found varied results (20% to 36%). Barde et al.[11] found 20.2% IgM positivity in dengue cases. In this study, IgM positivity was 24.2%. A few serological surveys for antibodies to dengue virus have been conducted previously in India. They revealed widespread occurrence of dengue infection all over India. Smithburn et al.[22] in a study from Nagpur and surrounding areas found that 20% to 40% sera of patients showed antibodies against dengue viruses.

Vijayakumar et al.[21] stated that dengue IgM positivity is an indicator of unusual dengue virus activity and reported that IgM positivity has been increasing since 1999 to 2003, mostly in children. Children are more vulnerable to dengue infection as also reported by Cherian and coworkers.[23] They also reported a significant increase in the percentage of positive samples for dengue IgM and IgG antibodies suggestive of secondary infection, only in adults and not in children. They also observed high mortality in children when compared with adults. Vijayakumar et al.[21] demonstrated 21% IgM antibody against dengue virus in children younger than 15 years when compared with 39% positivity in adults from Vellore as 26% as compared to decreased in adults to 11% positivity. In this study, IgM positivity in children was found to be 40.7% when compared with adults 19.35%. Our results are in accordance with the results of Vijayakumar et al.[21] in this respect. Findings of these studies show that primary infections by IgM positively against dengue are more common in children during epidemic and endemic periods when compared with adults. Wearing and Rohani[24] studied the Bangkok dengue epidemiological pattern and postulated that this pattern of more IgM positivity in children and less in adults, which was consistent with a period of short-lived cross-immunity in adults. Findings of Vijayakumar et al.,[21] which demonstrated the consistent findings of combined IgM and IgG positivity against dengue in children aged less than 15 years are lower than adults, which also supports this cross-immunity phenomenon. In this respect, findings of our study are also similar to Vijayakumar et al. In this study, combined IgM and IgG positivity in children was 18.8% when compared with adults (38.8%), signifying that adults are comparatively more subjected to combined primary and secondary infections when compared with children.

In order to provide timely information for the management of patients and early public health contact dengue outbreaks, it is important to establish a diagnosis of acute dengue virus infection during the first few days after manifestation of clinical symptoms. Kumarasamy et al.[25] stated that early laboratory diagnosis of dengue virus infection still remains a problem. They suggested three basic methods used by most laboratories for diagnosis of dengue viral infection, which are virus isolation and identification, detection of virus genomic sequence by nucleic amplification assay (RT-PCR), and detection of dengue virus-specific IgM antibody by Mac ELISA. Virus isolation and characterization are considered as gold standard tests for acute dengue virus infection. The limitation in this test is that it is expensive and takes 6–10 days. RT-PCR is also an expensive test and not widely available in many hospitals. The limitation of IgM antibody is that it appears after 4–5 days of infection and second for its efficacy. Paired sera test is advisable to see the rising titer. In a study of 192 dengue cases found, RT-PCR positivity was found in only 25% cases, while another study of 89 cases from Jabalpur in 2010 reported only one (1.15%) RT-PCR–positive case in their study.[11,25] In our study, a total of 12 RT-PCR tests were conducted in 38 cases, which were NS1- and IgM-positive and 10.5% RT-PCR positivity was recorded. RT-PCR results depend upon stagers of acute infection; this explains variability of positivity by different workers. If the test done early in the acute phase when the virus particles were present in serum, high positivity will be found, but when the test is done late in acute phase, reduced positivity is recorded, as, by that time, virus particles will be removed from circulation. Kumarasamy et al.[25] compared various methods for early acute phase diagnosis with overall higher sensitivity rate that advocates the NS1 antigen, which gives a higher positive detection within the first 4 days of illness. They also observed that NS1 Ag-captured ELISA gave a significantly higher detection rate in acute primary dengue than a secondary dengue; they also opinioned that the sensitivity rate of IgM assay for early diagnosis of dengue were poor in the first 3 days of illness. This firmly advocated that NS1 Ag-captured ELISA as the test for choice for patients suspected of acute illness, with a word of caution that there can be possibility cross-reactivity with NS1 antigens of other *Flavivirus*.

The scenario on the dengue status from various parts of India is not different. Incidence of seasonal variations from various parts of India is directly proportional to the increased postmonsoon vector, which is also associated with irregular

and indiscriminate heavy rain fall in different parts of the country. Over the years, there was a significant overall increase in dengue seropositivity in children and not in adults. Not only seasonal variation, but dengue also shows rural, urban, and other demography patterns with various parts of India and is fast emerging as a major health concern in India. Molecular studies on the circulating serotypes and their genotypes may be of help in addressing the probability of DHF/DSS incidence in future.

Conclusion

Involvement of many laboratories in the diagnosis of dengue coupled with general awareness among the public and constant vigilance by the health-care officials could go a long way in combating dengue, especially in the prevention of large outbreaks by monitoring dengue viral activity by serological and molecular tests.

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How to cite this article: Gupta D, Jain SB, Jain B, Ranjan KP. Comparative analysis of clinicopathological findings with immunological and molecular tests for early diagnosis and confirmation of dengue in a tertiary-care hospital in Gwalior. Int J Med Sci Public Health 2016;5:171-175

Source of Support: Nil, **Conflict of Interest:** None declared.