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Rapid detection of dengue virus by multiplex real time PCR at SMS medical college & attached Hospitals, Jaipur

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Abstract
Objective: To determine the magnitude of Dengue virus in patients presenting with Dengue like illness (DLI) during the post-monsoon period.
Materials and methods: A total of 100 blood samples of patients having DLI, attending SMS Medical College and attached hospitals were collected and processed for Dengue RT-PCR during the month of October 2017.
Results: Of the 100 patients enrolled in the study, overall 36(36.0%) patients were positive for dengue by RT-PCR were negative. In the group of positive patients, 24 (66.67%) whereas in the group of negative patients, 46 (71.88%) were males, with no statistically significant differences between the two groups. The age group with the highest positivity for Dengue was in adults between 21 and 30 years old (30.55%), followed by juveniles up to 20 years old (22.22%).Fever was seen in almost all cases. In dengue infection, common symptoms included myalgia (25%), joint pain (33.33%), headache (41.67%), and cough (13.89%).
Conclusion: In this study, we intended to bring attention on the presence of viral etiologies of febrile illnesses in Rajasthan. Our results suggest that DENV virus should be considered in differential diagnosis of patients with fever. For the laboratory diagnosis of DENV infection molecular assays are more sensitive in the early stages of illness. Moreover in multiplex PCR other agents could also be tested simultaneously thus adding to diagnostic armamentarium leading to rapid and early diagnosis and management of febrile illnesses.

Keywords: dengue, RT-PCR, Jaipur

1. Introduction

Arthropod-borne viruses or arboviruses are one of the major public health problems worldwide. Dengue is the most important arthropod – borne viral infection of humans [1]. DENV (Dengue Virus) is single-stranded, positive-sense Ribonucleic Acid (RNA) virus. DENV belongs to the family Flaviviridae and genus *Flavivirus* of which there are five known serotypes (DENV 1–5). The DENV genome encodes three structural (C, prM, and E) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins [2]. The World Health Organization (WHO) estimates more than 2.5 to 3 billion people are at risk of infection in over 100 countries in urban, peri-urban and rural areas of the tropics and subtropics [3]. Dengue ranks as the 11th highest cause of morbidity and 10th highest cause of mortality from neglected diseases. 50-100 million dengue infections occur each year worldwide, with at least 500,000 cases of DHF or Dengue shock syndrome (DSS), which require hospitalization [4]. Case-fatality rates for untreated DHF or DSS can be as high as 30-40%. The risk of DHF or DSS is highest in areas where two or more DENV serotypes are transmitted [5]. In many countries of South-East Asia around 95 per cent of the dengue cases are among children of less than 15 years of age [6].

Aedes aegypti and *Aedes albopictus* mosquitos are vectors for DENV. The majority of DENV infections affect people residing in endemic areas, which include most of the tropical and subtropical regions in the world [7]. In most studies, highest numbers of Dengue cases were detected during the month of October followed by the month of November, which is the post-monsoon period. The stagnant fresh water during the rainy season (June to September) favors the breeding of the vector mosquitoes [8]. Thousands of cases of dengue fever are reported every year in North India. Dengue fever epidemics have occurred in North India in 1967, 1970, 1982, 1988, 1996, 2003, 2006 and 2010 [9].

Accurate and rapid diagnosis is critical, as this will facilitate treatment in case of atypical presentations and complications, early distinction between dengue and other febrile illness could help identify patients with signs of DHF that could develop into life-threatening DSS. Therefore, the present study was planned to study magnitude of Dengue virus infection in Dengue like illness (DLI) cases using multiplex RT-PCR in patients attending SMS Medical College hospitals, a tertiary care center where patients come from all over Rajasthan.

2. Materials and Methods

The present study was an observational study & carried out during month of October 2017 at the Department of Microbiology & Immunology, SMS Medical College, Jaipur (Rajasthan) in 100 clinically dengue like illness patients of fever with myalgia, rash, and/or arthralgia. The samples which were found leaking, inadequate quantity, received through improper cold chain maintenance during transportation and samples collected in improper containers were rejected. Five milliliters of whole blood was withdrawn from each patient by venipuncture and transferred to plain vial for Denv RT-PCR. Samples were transferred to the laboratory at 2-8 °C as soon as possible.

Demographic data (such as age, sex, in-patient, out-patient status) of the patients, clinical history (such as duration of fever) and associated symptoms like arthralgia, rashes, gastrointestinal symptoms etc. were also recorded.

Sample processing and storage

All samples were centrifuged at 1500 rpm for 10 minutes, serum was separated and aliquoting was done in two tubes one for processing and another one for storage at -70^{0}C till further use. Each sample was processed in accordance with established Standard Operating Procedure (SOPs) in the laboratory. Nucleic acid was extracted from serum by automated nucleic acid extraction system and this nucleic acid was used for detection of DENV by real time RT-PCR.

Reverse transcriptase-Real time polymerase chain reaction (Real Time RT-PCR)

Total nucleic acid was extracted from 200µl of serum sample using a Nucleisens Easy Mag semi-automated nucleic acid extraction system (Biomerieux) and was eluted in a volume of 60µl.The one-step multiplex real-time RT-PCR assay for DENV was done using AgPath one step RT PCR kit and customized primers and probes [10]. To set up a RT-PCR reaction, 5 µL of RNA were mixed with the following reagents: 4.5 µL of nuclease-free H_2O, 12.5 µL of 2× RT PCR Buffer, 0.4 µL of each forward and reverse primers specific for DENV, 0. 2 µL of the Taqman probe specific for DENV and 1 µL of 25 X RT-PCR Enzyme Mix to a final reaction volume of 25 µL. Individual reactions were run in 8-tube optical strips and placed in the ABI 7500 FAST Dx Real Time PCR instrument (Applied Biosystems). Thermocycling parameters were as follows: reverse transcription (RT) at 50°C for 30 min, RT inactivation at 95°C for 10 min and fluorescence detection for 45 cycles of 95°C for 15 seconds and annealing at 55°C for 30sec. Amplification curves with Ct values >35 were considered negative.

For each sample:

Reagent	**Volume**
2X PCR buffer	12.5 µl
25 X RT-PCR Enzyme Mix	1.0 µl
Assay Mix (For DENV)	2.0 µl
Nuclease free water	4.5µl
…….…….	
Total volume	20 µl

The master mix was dispensed in real time fast reaction tube strips in 96 well plate format. 5µl of extracted RNA was added to each well. 5 µl of nuclease free water was added to the Non template Control wells (NTC). After addition of RNA sample, tube strips were covered with cap strips, pressed tightly to avoid evaporation during PCR, and briefly centrifuged. Individual reactions were run in 8-tube optical strips and placed in the ABI 7500 FAST Dx Real Time PCR instrument (Applied Biosystems).

Thermal cycling conditions

Thermocycling parameters were as follows: reverse transcription (RT) at 50°C for 30 min, RT inactivation at 95°C for 10 min and fluorescence detection for 45 cycles of 95°C for 15 seconds and annealing at 55°C for 30sec. Amplification curves with Ct values >35 were considered negative. Customised primers and probe for the detection of DENV and CHIKV were used [10].

3. Results

During October 2017, 100 blood samples were collected from patients presenting with Dengue like illness (DLI) of at SMS Medical College and attached Hospitals, Jaipur and sent to DHR State Level VRDL, Advanced Research Laboratory, Department of Microbiology & Immunology, SMS Medical College, Jaipur (Rajasthan) and evaluated in this study. The mean age of the patients was 27.94 years (SD ± 17.50 years); 70 (70%) were males and 30 (30.0%) were females. (Table 1)

Table 1: Demographic details of DENV infection among patients presenting with Dengue like illness (N=100)

Variables	**DENV infection Negative (N = 64)**	**DENV infection Positive (N = 36)**
Male N (%)	46 (71.88%)	24 (66.67%)
Female N (%)	18 (28.13 %)	12 (33.33%)
Age years (Mean±SD)	26.74 ±17.64	28.14±17.77
0-10	12 (18.75%)	5 (13.89%)
11-20	14 (21.86%)	8 (22.22%)
21-30	17 (26.56%)	11 (30.55%)
31-40	8 (12.5%)	6 (16.67%)
Older than 40	13 (20.31%)	6 (16.67%)

Of the 100 patients enrolled in the study, overall 36(36.0%) patients were positive for dengue by RT-PCR (days of illness, mean±SD: 2.85±1.33) and 64 (64%) were negative. The mean age of the patients who were negative was 26.74 years (SD±17.64 years), and that of the patients with confirmed infection was 28.14 years (SD±17.77 years). In the group of positive patients, 24 (66.67%) whereas in the group of negative patients, 46 (71.88%) were males, with no statistically significant differences between the two groups. The age group with the highest positivity for Dengue was in adults between 21 and 30 years old (30.55%), followed by juveniles up to 20 years old (22.22%). (Table 1).

Fever was seen in almost all cases. In dengue infection, common symptoms included myalgia (25%), joint pain (33.33%), headache (41.67%), and cough (13.89%). (Table 2)

Table 2: Clinical features of DENV infection positive patients (n=36)

Fever	**36 (100%)**
Fatigue	3 (8.33%)
Myalgia	9 (25.0%)
Joint pain	12 (33.33%)
Headache	15 (41.67%)
Retro-orbital pain	3 (8.33%)
Nausea	1 (2.78%)
Vomiting	2 (5.55%)
Diarrhea	0
Abdomen pain	2 (5.55%)
Anorexia	3 (8.33%)
Jaundice	2 (5.55%)
Cough	5 (13.89%)
Bleeding	1 (2.78%)
h/o of insect bite	12 33.33%)

4. Discussion

Dengue is globally important arboviral infections. It has been known to be endemic in India for over two centuries, thought to be benign and a self-limiting disease[25]. The conventional approach to diagnosis is detection of IgM antibodies specific to DENV. However patients come in varying stages of infection and as a result in early presenters the diagnosis may be missed by only IgM detection.

Present study was carried out to detect Dengue infection using PCR for detection of infection by DENV. The endemicity of DENV in North West India is well established and the vector is also present ubiquitously in our country. Hence we planned to study the infection due to DENV in patients presenting with DLI, to determine the magnitude of dengue. Testing the suspected patients for the dengue virus is important for proper patient management and for epidemiologic reasons.

In our study, among the 100 patients enrolled, 36 (36.0%) were positive for DENV. Dengue positivity similar to us was also reported at Tanzania [11] (38.2%), St. Martin [12] (38%) & Sri Lanka [13] (37%), Varanasi [14] (30%), Yemen [15] (29%), and Pune [16] (26.4%). Dengue positivity higher than us was reported at Delhi [17] (70%), Mumbai [18] (59%) and Maharasthra & Odisha [19] (47%), in India. The positivity varies from place and to place and is different in a place in different year, this happens due to multiple factors like global population growth and the associated unplanned and uncontrolled urbanization, lack of effective mosquito control, resurgence of epidemic dengue, international travel etc [20].

In present study, the mean age group affected was 21-30 years in DENV infection as this group is maximally involved in outdoor activities and being occupationally active has higher chances of exposure to arthropod-borne viruses. Similar observations were reported by other authors from India, Delhi [21]. They reported that the mean age of dengue patients was 27 years and the maximum belonged to the 21-30 year age group (32.44%). Similar trend was observed from Delhi [21], Hyderabad [22] and West Bengal [23].

The males (66.67%) outnumbered females (33.33%) in DENV positivity, similar pattern was observed by other researchers at Delhi [24], Jaipur [25] and Tamil Nadu [26].

In the present study was done during the month of October i.e. post monsoon season, in which highest number of Dengue cases occurs as reported from most of the studies from Karnataka [27] and Delhi [24]. This is due to fact that this season is very favorable for breeding of the vector, Aedes aegypti and higher humidity lengthens the life span of mosquitoes and the temperature shorten the extrinsic incubation period.

In present study commonest symptoms observed were fever (100%), headache (39.89%), joint pain (32.63%), myalgia (25%), and retro-orbital pain (8.4%) in patients infected with DENV. Similar findings were reported at West Bengal[23] and Mumbai [18].

RT-PCR positive indicate acute infection, PCR is reported to be positive up to 5 days of illness (days of illness, mean ± SD: 3.44±3.10). DENV infection should be on the differential diagnosis at the initial patient presentation in the patients with acute febrile illness especially in endemic regions.

Molecular assays are more sensitive for diagnosis in the early stages of illness (2–5 days after onset) when antibodies are not detected. However, in the later stages of illness, the sensitivity of molecular methods decreases due to the onset of a brisk immune response and corresponding reduction in viral load. At this stage, the IgM ELISA is a more sensitive diagnostic test.

An ideal diagnostic test should meet certain key criteria: affordability by those at risk of infection, specificity, sensitivity, ease of use, rapid results, little reliance on equipment, and delivery to those in need [28].

A one-step multiplex real-time RT-PCR assay can simultaneously detect and quantitate RNA for all DENV serotypes. Cecilia [10] *et al*. reported a sensitivity of 100% for DENV, while the specificity was 100% for DENV when compared to conventional RT-PCR. Pongsiri [29] *et al.* reported an assay sensitivity of 97.65% and specificity of 92.59% when compared to conventional RT-PCR. The one step process reduces the chance of contamination and there is lack of cross-reactivity between related Flavivirus groups and DENV [30]. Multiplex RT-PCR can simultaneously detect multiple pathogens thus reducing cost and time [31]. Moreover besides DENV, CHIKV and ZIKV virus has also been added in CDC trio-plex to detect all three agents simultaneously as they have similar signs and symptoms, incidence of positivity and same vector too [32].

Accurate and early diagnosis of DENV infection would help in appropriate management. Greater awareness of the prevalence of arboviral infections among clinicians and the availability of a reliable and affordable diagnostic test would improve patient management and contribute to a more robust assessment of disease burden.

Limitations

In our study, detailed haematological and biochemical parameters were not noted and compared with molecular profile, nor was patient's treatment outcome followed. Also serological methods were not included. Nevertheless, we could timely diagnose and identify the magnitude of DENV infection in DLI patients.

5. Conclusion

In this study, we intended to bring attention on the presence of viral etiologies of febrile illnesses in Rajasthan. Our results suggest that DENV virus should be considered in differential diagnosis of patients with fever. Furthermore, we found that these diseases were manifested with similar symptoms hence clinical diagnosis alone was not sufficient to discriminate them.

For the laboratory diagnosis of DENV infection molecular assays are more sensitive in the early stages of illness (2–5 days after onset) but in the later stages of illness, the sensitivity of molecular methods decreases due to the onset of a brisk immune response and corresponding reduction in viral load. At this stage, the IgM ELISA is a more sensitive diagnostic test.

Early detection can help in initiating appropriate and timely intervention and avoid misuse of antibiotics and the cost related to the treatment. Moreover in multiplex PCR other agents could also be tested simultaneously thus adding to diagnostic armamentarium leading to rapid and early diagnosis and management of febrile illnesses.

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