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Original article:

Comparison of NS1Ag detection by ELISA and Real Time RT–PCR for rapid diagnosis of dengue infection in acute phase

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Abstract

Background: Dengue fever is endemic in India and Punjab is one of the hotspots for it. Despite this the understanding of the incidence of disease as well the prevailing serotypes of dengue virus is lacking.

Objectives: The present study was conducted to compare NS1Ag detection by ELISA and Real Time RT–PCR for rapid diagnosis of dengue infection in acute phase among the patients admitted with clinical suspicion of dengue and to identify the dengue virus serotypes prevalent in this demographic area in 2017.

Methods: Detection of dengue is done by NS1Ag ELISA in patients having history of fever is less than 5 days. Out of 1025 NS1Ag ELISA positive samples, were selected for serotyping by real-time RT-PCR (n=70).

Results: Our study showed that serotype DEN-3 was predominant during this outbreak. The concordance between NS1Ag ELISA detection and real time PCR was found to be 100% for the number of samples to be tested (n=70).

Conclusion: Serological methods are unable to detect the infection during the early phase of the disease. Thus there is a need for rapid and sensitive methods for detection of DENV early in the course of infection for better patient management. NS1Ag ELISA and real time RT-PCR were found to be rapid convenient and efficient test for diagnosis of dengue fever in acute phase.

Key words: Dengue fever, Dengue Haemorrhagic Fever, Dengue Septic Shock, ELISA, RT PCR

Introduction

Dengue fever, the most important mosquito-borne viral infection, is prevalent in most parts of the tropical and subtropical areas and is responsible for high mortality rate as compare to any other arboviral disease in humans. Presently, about 40% of the world's population is at risk and there are 50–100 million cases every year. The case-fatality rate for DHF varies from country to country and is more prevalent among children and young adults with approximately 25,000 deaths each year. [1]In view of the high mortality rate and to reduce the disease burden, it is essential to have a rapid and sensitive laboratory tests for early detection of the disease. The major diagnostic methods currently available are viral culture, viral RNA detection by reverse transcriptase PCR (RT-PCR) and serological tests such as an IgM MAC-ELISA. The MAC-ELISA, which is a commonly used assay, has a low

sensitivity in the first four days of illness and there is requirement of paired sera at acute and convalescent phase, to improve the accuracy of the diagnosis. [3,4]

Rapid serological tests such as IgM or NS1Ag ELISA with a single serum sample do not furnish information about the circulation or the changing patterns of the serotypes of the virus. It is crucial to determine which serotypes of DENV are circulating since previous infection with one of the four serotypes can be an important risk factor for developing DHF-DSS upon infection with a heterotypic serotype. Therefore, serotyping with molecular methods can help us in acute phase of infection. [5]Molecular methods based on PCR offer a suitable alternative to conventional virus isolation. Several PCR based methods for detecting DENV nucleic acid in the serum have been reported, the most widely used test is the gold standard RT-PCR . [11,32] So, the rationale of this study is to compare the NSI Ag detection by ELISA which is commonly used diagnostic tool in our VRDL with newly standardized Real-time RT-PCR assay for rapid diagnosis and serotyping of dengue viruses in acute phase of infection.

Objective

The present study will compare the NS1Ag Detection by ELISA and Real Time RT–PCR for rapid diagnosis and serotyping of dengue infection in acute phase of dengue virus infection in a tertiary care hospital in Patiala region of Punjab.

Methods

Ethical approval of Institutional Ethics Committee was taken prior to beginning of the study.5 ml blood samples was collected aseptically from patients who were clinically suspected to be suffering from dengue fever due to presence of any or all of-fever/headache/myalgia/retro orbital pain/ rash/ hemorrhagic manifestations in the acute phase of their illness (1–5 days) and were transported to Viral diagnostic laboratory, Department of Microbiology GMC Patiala, India. Serum was separated aseptically from the clotted blood and was stored at –70°C until further processing.

Dengue NS1 antigen detection was done in serum samples by using In-Bios DENV "two-step" sandwich-type NS1 antigen ELISA (In-Bios International USA) as per manufacturer's instructions. Dengue IgM ELISA was done only on the serum samples from patients with duration of fever ≥5 days. The kit used was the μ-capture dengue IgM ELISA kit supplied by the National Institute of Virology, Pune; under the National Vector Borne Disease Control Programme. Manufacturer's instructions were strictly followed for performing the test and interpreting the results. The O.D. was measured at 450 nm using ELISA reader (Transasia Erba Mannheim, Germany) [23].

Out of 1025 NS1Ag ELISA positive samples, the samples which were 2ml in quantity, non-haemolytic, non-lipemic, maintained in cold-chain were selected for serotyping by real-time RT-PCR (n=70). Viral RNA for the real time RT-PCR was extracted from the NS1Ag ELISA positive serum samples by using the QI A-Amp Viral RNA mini kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions and stored at -70˚C until used for further experiments.The extracted RNA from clinical samples were subjected to DENV fourplex real time RT-PCR (Taqman) assay developed by Johnson et al, with slight modifications.[12] The primers and fluorogenic probes were purchased from Sigma and their sequences are listed in Table. Four sets of primer pairs and fluorogenic probes from which each set of primer pair was specific for one serotype, were designed for this study by following the methodology of Johnson et al,[12] with the exception of labelling of DEN-2 probe with different fluorophore at 5' end.

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The DEN-2 probe was labelled with TAMRA reporter dye at 5' end and BHQ1 at the 3'-end rest of the primers and probes used (DEN-1,3 and 4) were in accordance to the above reference. As mentioned in the referred manuscript the efficiencies of singleplex as well as fourplex DEN virus serotype specific real time RT-PCR assay were comparable. So, in present study we used the fourplex DEN virus serotype specific real time RT-PCR assay along with endogenous (RNase P) control reaction in singleplex. In fourplex reaction mixtures, final conc. of 200nM (each) of DEN-1 and DEN-3-specific primers, 100 nM (each) of DEN-2 and DEN-4-specific primers, 180 nM each probe and 0.5 µl of Super-Script III RT/Taq mix were used with addition of 5 µl RNA (isolated from NS1 ELISA positive samples) in a 25-µl volume of total reaction mixture. Individual reactions were run in 8-tube optical strips subjected to the following thermocycling parameters in ABI 7500 real time PCR system (standard mode), reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 10 min and 45 cycles of amplification with 95°C for 15 sec and 60°C for 1 min.[12] Amplification curves were evaluated by serotype and the threshold line placed above overt background signal intersecting the initial exponential phase of the curve for each serotype individually. A specimen is considered positive for either DENV1, 2, 3 or 4 if the amplification curve crosses the threshold line within 37 cycles. Amplification curves with C_T value >37 render erratically and are difficult to ascertain with increasing C_T values, therefore present unreliable results and were considered negative (according to CDC DENV1-4 Real Time RT-PCR assay).

Table: The sequence of Primers and Probes used in the present study

Primer	Reporter Dye	Sequence (5'-3')	Quencher
D1-F		CAAAAGGAAGTCGTGCAATA	
D1-R		CTGAGTGAATTCTCTCTACTGAACC	
D1-Probe	FAM	CATGTGGTTGGGAGCACGC	BHQ1
D2-F		CAGGTTATGGCACTGTCACGAT	
D2-R		CCATCTGCAGCAACACCATCTC	
D2-Probe	TAMRA	CTCTCCGAGAACAGGCCTCGACTTCAA	BHQ1
D3-F		GGACTGGACACACGCACTCA	
D3-R		CATGTCTCTACCTTCTCGACTTGTCT	
D3-Probe	TEXAS RED	ACCTGGATGTCGGCTGAAGGAGCTTG	BHQ2
D4-F		TTGTCCTAATGATGCTGGTCG	
D4-R		TCCACCTGAGACTCCTTCCA	
D4-Probe	CY5	TTCCTACTCCTACGCATCGCATTCCG	BHQ3
RP-F		AGATTTGGACCTGCGAGCG	
RP-R		GAGCGGCTGTCTCCACAAGT	
RP-Probe	FAM	TTCTGACCTGAAGGCTCTGCGCG	BHQ1

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Results

The present study was conducted in a period of July – November 2017 at tertiary care hospital Patiala. Febrile patients from Indoor and outdoor visiting Medical and Paediatric Departments, with H/O fever less than 5 days were subjected to investigations. Patients of all age groups, having a temperature of >38.5°C for >24 hours, and clinically diagnosed as having dengue fever were included in this study. Dengue NS1 antigen detection was done in serum samples by using DENV NS1 Ag ELISA (In Bios International USA) followed by serotyping was done by Real Time RT-PCR.

Maximum number of individuals were in age group of 21-30 years (27.18% of 1994) and minimum were in the age group of >70 years (0.95% of 1994) (Fig -1). Males were more (58.2%) as compare to females (42.8%). The male to female ratio was 1.39:1 (Fig -2). More number of cases were from urban areas; 82.8% as compared to 17.2% rural areas (Fig-3).1994 cases have history less than five days of fever and 1200 cases have history more than 5 days of fever. NS1Ag ELISA was performed on 1994 samples, out of which Dengue was positive in 51.4% of cases and 48.5% cases were negative for Dengue NS1Ag ELISA (Fig- 4). Maximum number of dengue positive cases were reported in October (n=734 positive cases) followed by November (n=657) and finally tapered by December (n=38). Percentage of positives cases peaked in November followed by October (70.1% of 936 cases and 50.5% of 1452 cases, respectively) (Table- 1). The most common symptom in dengue positive cases was fever (in 100% cases) followed by headache (99.7%) and myalgia (in 53.8% cases) (Fig -5). The most prevalent serotype in the given population is DEN-3 (54.2%), followed by DEN-1 (27.1%) and DEN -2 (5.71%) respectively. However, no case of DENV 4 serotype was detected in present study. Coinfection of DEN-1,3 was seen in 10% of cases followed by DEN1,2 (1.4%) and DEN 2,3 (1.4%) each (Fig -6). There was 100% concordance with serological existing diagnostic tool i.e. NS1Ag detection for the number of samples to be tested (n=70) (Table-2).

FIG 1: AGE-WISE DISTRIBUTION OF TOTAL NS1AG TESTED CASES

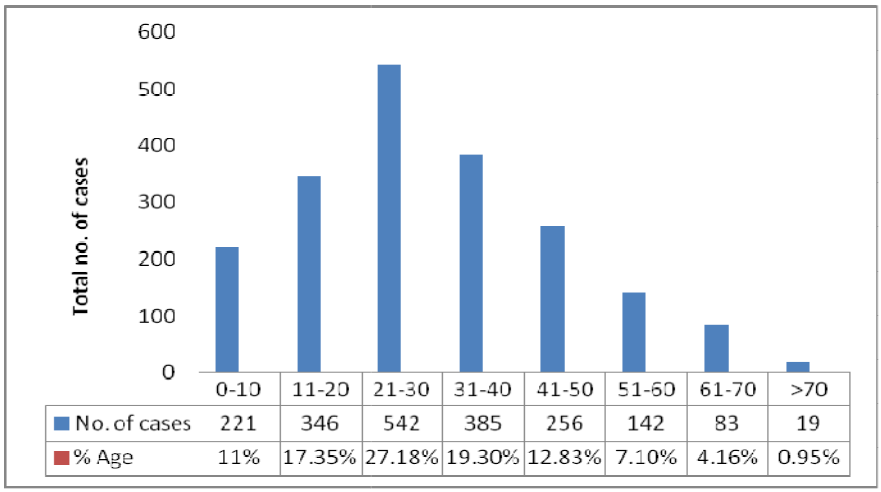

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Fig 2: GENDER WISE DISTRIBUTION OF TOTAL NS1Ag TESTED CASES

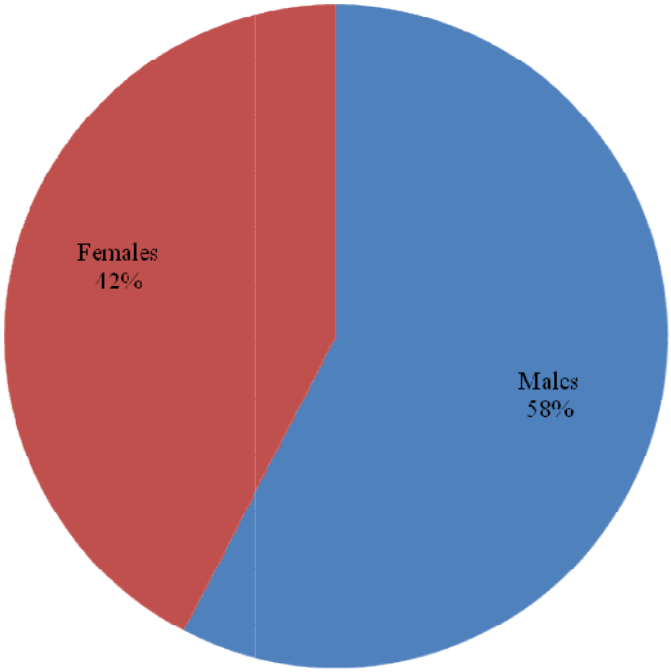

Fig 3: AREA WISE DISTRIBUTION OF TOTAL NS1Ag TESTED CASES

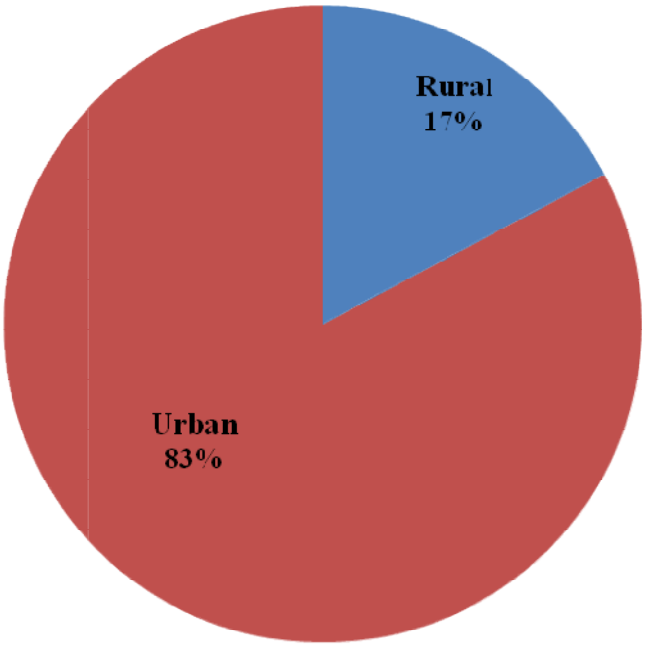

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Fig 4: TOTAL NUMBER OF SAMPLES TESTED ACCORDING TO HISTORY OF FEVER

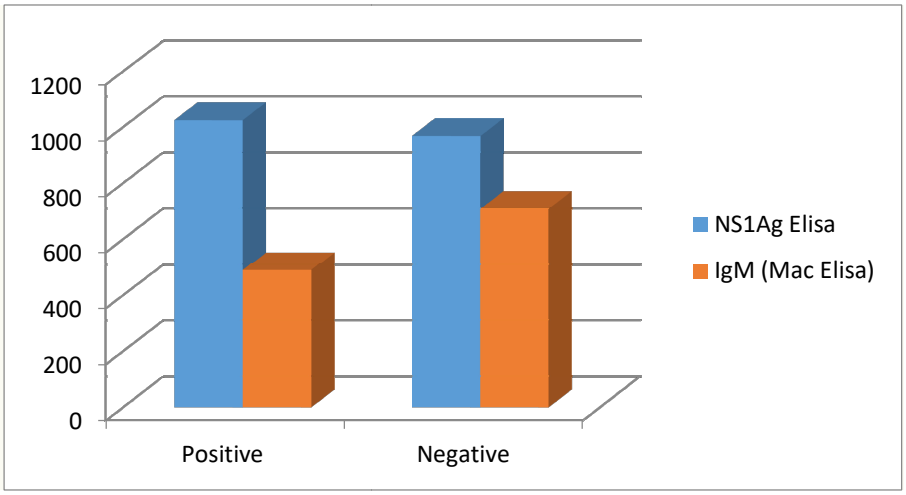
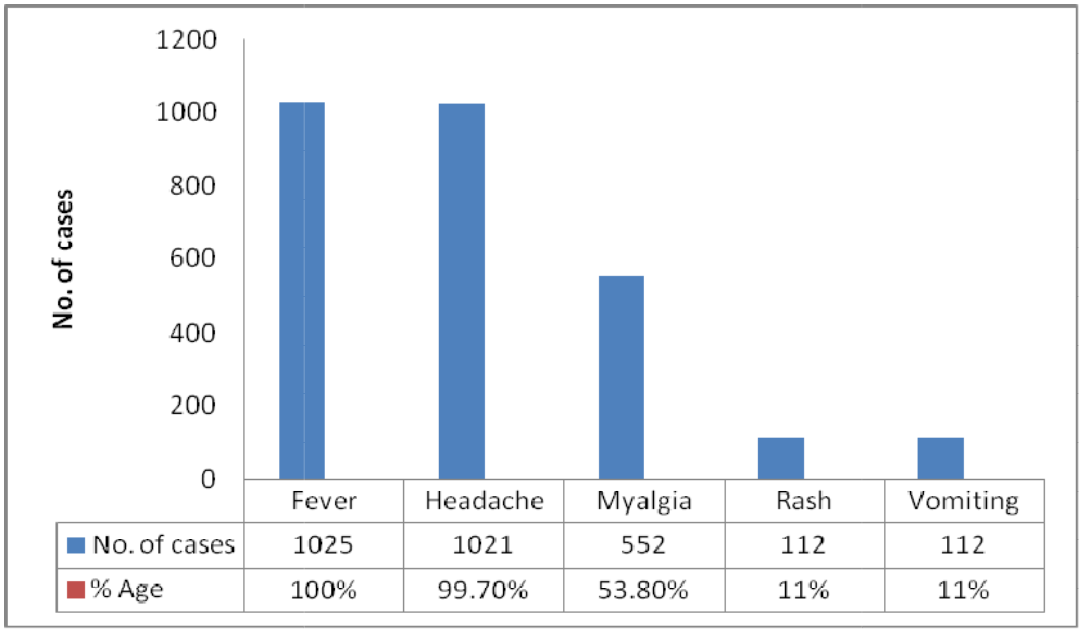


Table -1 Month wise distribution of dengue positive samples received from tertiary care hospital

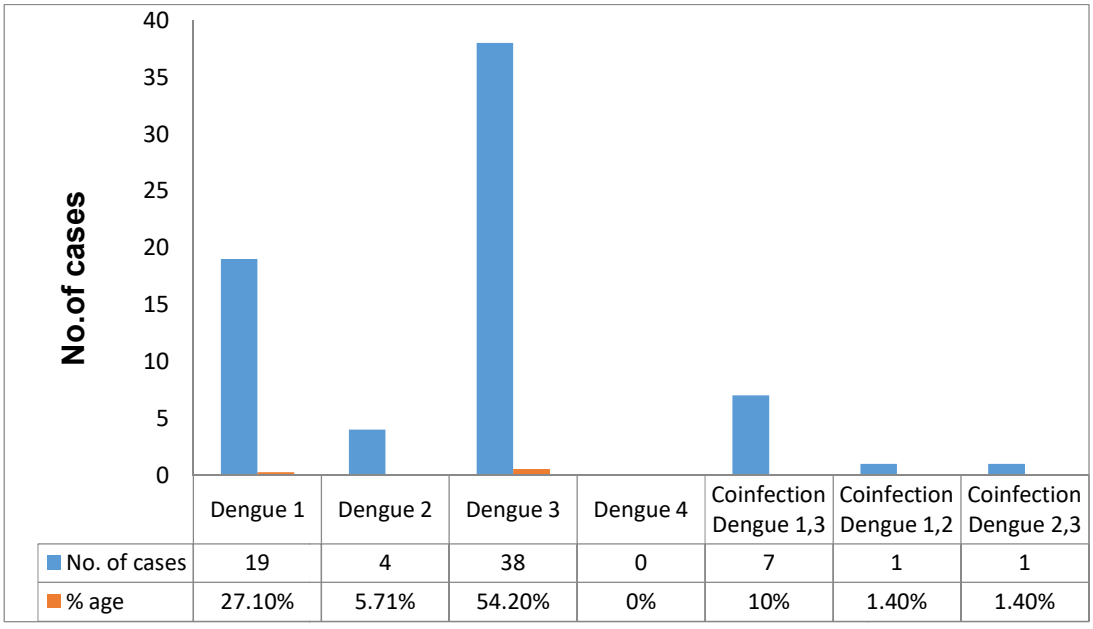
Month	No. of sample tested	Name of the test performed				%age Positive
		IgM Mac Elisa		NS1Ag Elisa		
		Total	Positive	Total	positive	
July 2017	202	104	00	98	01	0.49%
Aug 2017	305	157	06	148	03	2.95%
Sept 2017	315	163	69	152	25	29.8%
Oct 2017	1452	498	236	954	498	50.5%
Nov 2017	936	294	179	642	478	70.1%
Dec 2017	151	50	17	101	21	25.1%

Fig 5: DISTRIBUTION ACCORDING TO SYMPTOMS IN DENGUE NS1Ag POSITIVE CASES



	Fever	Headache	Myalgia	Rash	Vomiting
No. of cases	1025	1021	552	112	112
% Age	100%	99.70%	53.80%	11%	11%

Fig 6: DISTRIBUTION PATTERN OF VARIOUS SEROTYPES EXISTING IN THE PATIALA REGION OF PUNJAB (N=70).



	Dengue 1	Dengue 2	Dengue 3	Dengue 4	Coinfection Dengue 1,3	Coinfection Dengue 1,2	Coinfection Dengue 2,3
No. of cases	19	4	38	0	7	1	1
% age	27.10%	5.71%	54.20%	0%	10%	1.40%	1.40%

Table -2 Comparison of NS1Ag detection and DENV Fourplex Real Time RT-PCR

NS1Ag Detection	Real time RT-PCR		Concordance%
	Positive	Negative	
Positive	70	00	100%
Negative	00	00	
Total	70	00	

Discussion

India witnessed a massive wave of dengue fever, involving almost all states since 1996, but in year 2013 extreme north was massively effected. India also saw a doubling up of cases of dengue from 2014 to 2015 and the worst hit city was Delhi followed by Punjab. In Punjab 770 (2012), 4117 (2013), 472 (2014), 14128 (2015) and 10439 (2016) dengue cases had been reported along with following no. of deaths 9 (2012), 25 (2013), 8 (2014), 18 (2015) and 15 (2016) respectively because of dengue. [24, 25]

Dengue affects humans of all age-groups. The most common age group affected in this study was 21-30 years, followed by 31-40 years and 11-20 years respectively. This was comparable to other studies of Barror et al, Dar et al, and Gupta et al. The high number of cases in the adults and young patient age group implies that the disease is endemic in these regions. [26,27 28] The higher prevalence of dengue infection was noted among male than female patients. The male-to-female ratio was 1.39:1 which is comparable with the past studies conducted in Delhi and Ajmer. Male preponderance and the age group of 21-40 years indicate more transmission of dengue infections at work sites. [30, 31] More number of patients were from urban area (82.8%) as compare to rural area (17.2%). Similar were the results of study of Mahesh Kumar et al. This suggests that dengue is more an urban and semi urban infection because of overcrowding, which may become a cause for concern for health authorities. [31]The clinical profile of dengue revealed that fever; headache and myalgia were the most common presenting symptoms, with fever in all 1025 NS1Ag positive cases (100%). Classical DF is characterized by sudden onset of high grade fever, accompanied by headache, retro-orbital pain, myalgia, and thrombocytopenia. In our study, myalgia was seen in 53.8% and rash with thrombocytopenia in 11% cases. Thus, most of dengue cases were clinically indistinguishable from other febrile illnesses, and could be missed lacking the clinical suspicion and timely diagnosis. Similar results were reported by Neerja et al, Agarwal et al, Barror et al, Dar et al, Gupta et al, and Kaur H et al respectively. [9, 10, 26, 27, 28, 29]

To identify the seasonal variation of the disease, analysis of the data on monthly basis was done. The infection started spreading during the post moonsoon period i.e in October, peaked in November and slowly tapered by December which is similar to most of the previous outbreaks in India as reported by Hussain et al, Kaur et al, and

Bharaj et al [23, 29, 30]. It may be because this season is very favourable for high breeding of the vector, *i.e., Aedes aegypti*. Therefore, vector control measures should be started before monsoon to prevent the outbreaks of dengue. This will simultaneously solve the problem of other mosquito borne diseases like malaria, chikungunya, Japanese encephalitis and filaria.

Out of 1025 NS1Ag ELISA positive samples received from RH Patiala, the samples which were 2ml in quantity, non-haemolytic, non-lipemic, maintained in cold-chain were selected for serotyping by real-time RT-PCR (n=70). From 70 samples tested positive for dengue viral RNA by RT-PCR, 61 cases had single DENV serotype infection and 9 had concurrent infection with two DENV serotypes. Of 61 single infection cases, 38 were typed as DENV-3, 19 as DENV-1 and 4 were carrying DENV-2 serotype. Not even a single case of DEN-4 was reported in present study. DENV-3 seems to be dominating the present outbreak constituting 54.2% of the positive samples (n=70), followed by DENV-1 (27.1%). Thus, DENV-1 and DENV-3 were the most common serotypes observed during the outbreak. Similar were the results of studies of Vajpayee M, Gupta E, and Dash PK et al who reported DENV1 and DENV3 as the main serotype in an outbreak during 1999 and 2005 in New Delhi. [36, 28, 37] However in 1996, one of the largest outbreaks in North India occurred in Delhi and adjoining area with predominant serotype was DENV -2. [26, 27]

In present study, it was found that serotype DEN-1, 2 and 3 are co-circulating in Patiala region of Punjab. The overall prevalence of concurrent infections was 12.8% (9 out of 70 positive cases). Out of 9 cases of concurrent infections, DENV-1,3 co-infection was found to constitute 77% (7 out of 9) of the total concurrent infections. Other combinations of concurrent infection reported in the present study were DENV-1,2 and DENV-2,3 in 11% of cases (1 of 9) each. To the best of our knowledge, the present study is the first study from this region to report concurrent infection with different dengue virus serotypes. It is similar to the study done by Dar L et al, and Bharaj P et al, who reported concurrent infection of DENV-1 and DENV-3 in an outbreak in Delhi 2006. [3, 30] It has been postulated that concurrent infections with multiple dengue virus serotypes may influence the clinical course of the disease, and it is considered as the most important factor in the emergence of DHF. [30]

Conclusion

The geographical spread of four DENV serotypes throughout the subtropical regions of the world has led to larger and more severe outbreaks. In view of the high mortality rate and to reduce the disease burden, it is imperative to have a rapid and more sensitive laboratory assay for early detection of the disease.

To conclude, NS1Ag ELISA and real time RT-PCR were found to be rapid convenient and efficient test for diagnosis of dengue fever in acute phase and the diagnosis could be made as early as within 5 days of onset of fever. Early diagnosis of DENV infection can improve clinical outcomes by ensuring close follow up, initiating appropriate supportive therapy and raising awareness to potential of haemorrhage and shock. This will also reduce the time between the 1st case detected and the notification to public health departments to prevent the disease.

Acknowledgments

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