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Strategy to Diagnose Dengue Virus Infection in Local Settings: An Experience

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Results : All the 85 serum samples gave positive results on one step multiplex Real Time RT PCR and also gave bands on 2% agarose for dengue serotype 2, 3 and 4. Immuno fluoresent staining was also positive for dengue serotype (2).

Conclusion : The results of this study shows that both immunofluorescence technique as well as one step multiplex Real Time RT PCR are efficient and rapid techniques for diagnosis and for research purpose equally, if it would be fully establish in local setting. Along with these finding we also found that the major dengue virus infections in year 2012 were of dengue serotype 4, while dengue serotype 2 and dengue serotype 3 were present in very small percentage i.e., 10% and 6% respectively.

Keywords : DF (dengue fever), DHF (dengue hemorrhagic fever), DSS (dengue shock syndrome), RT PCR (reverse transcriptase polymerase chain reaction).

I. Introduction

Dengue fever is caused by a virus which is transferred through a vector called mosquito; and four serological types of dengue virus circulating in our environment are: DEN-1, DEN-2, DEN-3, and DEN-4 that causes dengue infection. Dengue fever is very valuable Arbovirus disease which causes disease in humans, and World Health Organization (WHO) indicates that its extent of effect has risen with part of thirty over the preceding fifty years (2). DENV is transferred towards humans through *Stegomyia aegypti* (previously *Aedes aegypti)* mosquitoes and broad variety of signs appear, as of mild disease (dengue fever) to a severe hemorrhagic type (dengue hemorrhagic fever) (2, 3). Genus of Dengue viruses is Flavivirus and family is Flaviviridae. Dengue viruses have single stranded RNA and are enveloped. The size of RNA genome is almost 11 kilo base and the RNA encodes a three structural proteins: E (envelope protein), M (membrane protein), and C (core protein), the large poly protein precursor are produced by RNA genome, which is co translationaly processed by virus encoded proteases and host cell to produce individual dengue virus protein. The NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5 are nonstructural (NS) proteins (2). DHF-DSS is a most important reason of hospitalization and death amongst children in Southeast Asia, where more than one million cases were recorded between 1987 and 1989. Over 30,000 deaths due to DHF-DSS in children have been reported worldwide since 1950 (4). DF is an infection of youth, in Southeast Asia. There is, though, confirmation to recommend that the occurrence of DHF among adults is rising (5). Dengue virus infection epidemics are observed periodically all over the tropics and dengue is endemic in Southeast Asia and India. In recent times it has become endemic in Pakistan, with cases observed in main hospitals all year around. A current study that was under taken in a tertiary care hospital in Pakistan illustrated that cases of DHF and DSS are on the increase (6). In Pakistan, the initial known report was in 1985 (7), whereby dengue virus serotype 2 was isolated for encephalitis in a sero epidemiological study. The initial main outbreak was testimony in Karachi in 1994-95 (8).

Hence, to stop and manage the development of dengue virus infection the World Health Organization has recommended the augmentation of active and accurate laboratory-based surveillance for early reporting of dengue virus infections to the public health authorities (9). In DHF and DSS epidemics, quick analysis of the serological type(s) within person having dengue disease is significant (10). Analysis is essential hence proper preclusion, treatment, as well as management measures are able to be started as well as exact epidemiologic facts be able to be retained (4). Dengue virus infection diagnosis on the basis of medical conditions is not consistent; therefore analysis must be definite through learning of laboratory (11).

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Infection with Dengue virus is now identified by means of numerous biological experiments: on mosquito cells virus isolation, viral RNA discovery via reverse transcription-PCR (RT-PCR) or experiments of serology, for example immunoglobulin M (IgM) capture enzyme-linked immunosorbent assay (MAC-ELISA). Commercial MAC-ELISA kits are accessible, but they cannot be utilized for early diagnosis, because IgM cannot be visible until 5 to 10 days later than the start of infection in cases of primary dengue virus infection and until 4 to 5 days later than the start of secondary infections (2). Immunofluorescence experiments used in the precedent, novel techniques relating enzyme conjugates for example peroxidase as well as phosphatase with combination to either monoclonal or polyclonal antibodies are very much progressed (12). Recognition of the DEN virus is commonly skillful by means of immunofluorescence methods along serological type-specific monoclonal anti-DEN antibodies on infected cells or vector head crushes (13).

II. Materials And Methods

There were 85 serum samples and 3 whole blood samples collected from suspected dengue patients, from different hospitals and laboratories of Karachi city during the months of August to November 2012. Initially 03 whole blood samples were subjected to Immunofluorescent staining technique, this technique was used for the identification and detection of dengue viral antigen. For performing immunofluorescent staining technique we followed the protocol of Sirichan Chunhakan et al; 2009 (14).

RNA was isolated from 85 serum samples from suspected dengue patient by using the extraction kit (viral gene spinTM) from intron. The isolated RNA were amplified by one step multiplex Real Time RT PCR. The purified RNA was amplified by using puRe Taq Ready-To-Go PCR Beads, (Amershan Bio sciences UK.). CFX 96TM Real Time C1000 Touch thermal cycler (Bio rad) was used for one step multiplex Real Tme RT PCR. 0. 6ul was taken from each primer and added in puRe Taq Ready-To-Go PCR Beads tube, then in this added 1ul M-MLV Reverse Transcriptase (Promega) and 5ul of M-MLV 5X Reaction buffer (Promega) and 7ul of isolated RNA. The final volume of 25ul was made by diluting this master mix reaction tube with diluents. Thermal cycle for dengue Real Time RT PCR was set. Initially the RNA was reverse transcribed to cDNA by providing it temperature of 55°C for 10 minutes, this was followed by one cycle of denaturation at 95°C for 8 min, next amplification was proceeded with 50 cycles at 95°C for 10 sec and one cycle at 60°C for 1 min (15). After that amplicons were subjected to 2% agarose gel in order to observe the size of amplified cDNA (complementary DNA) with the help of marker DNA (fermentas 1kb Ready-To-Use).

III. Results

A total of 85 serum samples and 03 whole blood samples were collected from suspected dengue patients, from different hospitals and laboratories of Karachi city during the months of August to November 2012. Out of 85 serum samples 17 serum samples were collected from Dow University of Health Sciences , 20 serum samples from Essa Laboratory and Diagnostic Centers , 28 serum samples from Imam Clinic and 20 serum samples and 03 whole blood samples were also collected from Memon Medical Institute as shown in Table 1. RNA was extracted from those suspected serum samples using viral gene spinTM. Isolated RNA was subjected to one step multiplex Real Time RT PCR and then processed on 2% agarose gel for electrophoresis. After performing all steps, it was observed that all 85 serum samples from different dengue suspected patients gave positive one step multiplex Real Time RT PCR results and also produced relative bands on agarose gel. The bands on 2% agarose gel electrophoresis of dengue cDNA gave almost equal sizes or lengths which were in the range of 1,500 bps to 2,000 bps as shown in Figure 3 and some were in the range of 2,500 bps to 3,000 bps as shown in Figure 4.

The molecular weight of isolated bands were in the ranges of 1,500 bps to 2,000 bps and 2,500bps to 3,000 bps as also mentioned by other scientist, and confirms the presence of dengue virus. It is important to note that majority of isolates were of dengue type 4 in about 84%, where as dengue serotype 2 was in 10% and dengue serotype 3 was in 6% only as shown in Table 2.

A total of 03 whole blood samples of dengue positive (IgG and IgM antibodies) serum patients were collected and preceded for immunofluorescence staining. This was the very first time we were trying to use immunofluorescence staining for the detection of this virus in Karachi, Pakistan we have to tried to establish each and every requirement and all the necessary equipments. That is why, we tried first with only three (100%) positive specimens and the results were promising. Our findings suggests that the DENV2 - infected monocytes were present in the slides as observed by using immunofluorescence microscopy and shown as green fluorescent objects in staining.

IV. Discussion

Molecular techniques are very useful and beneficial in a sense of detecting, identification and typing of dengue virus and also for epidemiological studies. Initially in this study, we tried to establish immunofluorescent technique in local setting but due to

lack of/ unavailability of required reagents in enough quantity, we had tried this technique only on three positive whole blood samples and get progressive results for DEN-2, it means that immunofluorescent technique is a easiest and rapid technique if we may establish it in local settings. Moreover we also detected, identified and evaluated that, which dengue serotype is commonly circulating in our community or environment. For that purpose along with immunofluorescence technique we also did one step multiplex Real time RT PCR. After performing one step multiplex real time RT PCR, we obtained positive results, means all 85 dengue serum samples from suspected dengue patients had RNA of almost equal length on 2% agarose that is in the range of 1,500 bps to 2,000 bps and very small in numbers the bands were in the range of 2,500 bps to 3,000 bps, it also suggests that majority of the 85 serum samples from suspected dengue patients shared almost the same serotypes in Karachi city during climate year of 2012.

Anna P. Durbin et al,. 2001(16) indicated that the size of dengue virus serotype 4 complete genome is 2,000 bps in another study Ching-Juh Lai et al, 1991 (17) showed that the full genome size of dengue virus serotype is 1343 bps, so it means that complete genome size of dengue virus serotype 4 is in the range of 1,000 bps to 2,000 bps while the size of dengue virus serotype 2 according to Stephanie Polo et al, 1997(18) is 3.2kb which is approximately 3,200 bps, Lewis Markoff et al, 2002(19), said that the size of dengue serotype 1 is 1-1.5 kb which is approximately 1000-1500 bps. According to Joseph E. Blaney JR, et al, 2004(20) dengue serotype 3 has approximately 2,000 bps, but according to our results, as we observed on 2% agarose gel about 84% bands were in the range of 1,500 bps to 2,000 bps, and dengue serotype 2 was present in about 10% and dengue serotype 3 was present in about 6%, it means that the dengue serotype 4 which is about 84% was the major cause of dengue outbreaks in Karachi in 2012. According to Erum Khan et al, 2010(21) in the year of 2004 dengue serotype 2 was dominant and in year of 2005 the dengue virus serotype 3 was dominating. According to Muhammad Idrees et al., 2012 (22) in the year 2006-2009 both the dengue serotype 2 and dengue serotype 3 were co circulating and in 2011 dengue serotype 2 was dominating. Our results showed that in 2012 the dengue serotype 4 was more dominating than dengue serotype 2 and dengue serotype 3 and may be responsible of causing severe dengue infections i.e. dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) during that particular year. Situation for the coming years especially for the patients who had previously infected with the dengue infection and in case of secondary infection with any of the four serotype of dengue the resultant will be more severe that's why it is recommended to take serious measures to eliminate the dengue infection from our region and make maximum effort to prevent the people from dengue infection.

Secondly, one step multiplex Real Time RT PCR detected dengue viral RNA and reverse transcribed dengue viral RNA into complementary DNA (cDNA), this evidence showed that all 85 serum samples from suspected dengue patients had been collected in early phase, this is because RNA remains in human blood during only first five to six days of infection, after that immunoglobulin M (IgM) produces in blood and start to eliminate the dengue virus, then after immunoglobulin G (IgG) produces and further precede the elimination of dengue virus and that IgG stays in blood for long interval of time and give immunity to person with same serotype. That is why all 85 serum samples were diagnosed as early phase serum samples and had shared almost the same serotype of dengue virus in Karachi city in 2012. These results help in epidemiological study and aid in collection of surveillance data of dengue viral infection from Karachi city.

Karachi city is geographically distributed into 5 districts which are; central, south, east, west and malir district. As we collected the sample from different hospitals and laboratories of Karachi city which are located in different districts of Karachi city, the higher number of dengue samples were received from central district that were about 48 in number and lowest number of dengue samples were received from district east that were about 40 in numbers. So in this study we also find that in 2012 dengue serotype 4 was distributed in district central and east of Karachi city while other dengue serotype that are dengue serotype 2 and dengue serotype 3 were also circulating almost in all district but in very small numbers. The percentage of dengue serotype 4 from different district of Karachi was about 84% while dengue serotype 2 and 3 were only 10% and 6% respectively. As the other studies mentioned that in previous years dengue serotype 2 and dengue serotype 3 were dominating so, it may be possible that in coming next year all four serotype will co circulating in all districts of Karachi city and may result in more sever dengue infection. So, this is the time to get rid of dengue infection and save the people from sever dengue infection. We hope that this all data about dengue infection and distribution of dengue infection according to districts of Karachi city will be helpful in elimination of dengue infection.

Most important thing in any viral infection is the management i.e. identification and characterization of causative agent. If we identify and characterize viral disease then, it becomes easy to design strategy for elimination of that disease from the community or environment. This was the reason why we evaluated the immunofluorescent technique and one step multiplex real time RT PCR because they gave us very rapid results. One step multiplex real time RT PCR is very rapid, specific and sensitive method, as well as

immunofluorescent technique is also a very rapid and easy, these techniques are useful at both research and diagnostic level and by using these techniques we may easily typify the dengue virus and will help to determine further measures to eliminate dengue virus and related infections from our community or environment.

The results of this study shows that both immunofluorescence technique as well as one step multiplex Real Time RT PCR are efficient and rapid techniques for diagnosis and for research purpose and the immunofluorescent technique is very beneficial if it would be fully established in local setting and along these finding we also found by using immunofluorescent technique, one step multiplex Real Time RT PCR and agarose gel electrophoresis that the major dengue virus infections in year 2012 was of dengue serotype 4, while dengue serotype 2 and dengue serotype 3 were present in very small percentage. Geographically dominating dengue serotype 4 was present in most of the district of Karachi city while, dengue serotype 2 and dengue serotype 3 were also present in almost all district of Karachi city. It is an alarming situation for coming years because, it may be possible that in next monsoon season all dengue serotype1-4 will be circulating and an outbreak will happen which may cause co-infections with more severity so before the monsoon season we should take some measures for elimination of dengue infection and prevents the lives from severe dengue infection. This result may be of help in eliminating dengue infection in the next coming years.

Our study provides very beneficial information on immunofluorescent technique for the diagnosis and research purpose, and its use for diagnosis of dengue and on the basis of this study we suggests that this technique will be very helpful in future in many ways. And by using both techniques the data we found will be helpful for the further epidemiological study and our study will also be helpful in elimination of dengue virus infection from our community by making different strategies and management for dengue infection and it may also be helpful in future work on vaccine preparation.

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Table 1 : Collection and area wise distribution of serum samples

Hospital/Laboratories Name	District of Karachi	No. of Samples (100%)
Dow University of Health Sciences (DUHS)	District East	17 serum samples (19%)
Imam Clinic	District Central	28 serum samples (31.81%)
Memon Medical Institute (MMI)	District East	20 serum samples and 03 whole blood samples (26.13%)
Essa Laboratory And Diagnostic Centre	District Central	20 serum samples (22.72%)

Table 2 : Distribution and Percentage of Dengue Serotypes

Total no. of Samples	Negative Serum Samples	Positive Serum Samples			
		Den type 1	Den type 2	Den type 3	Den type 4
85 serum samples (100%)	0 (0%)	0 (0%)	9 (10%)	5 (6%)	71 (84%)

Table 3 : Immunofluorescence Positive Blood Specimens

Total no of samples	Negative serum samples	Positive serum samples			
		DEN-1	DEN-2	DEN-3	DEN-4
03 Blood Samples (100%)	0 (0%)	0 (0%)	03 (100%)	0 (0%)	0 (0%)

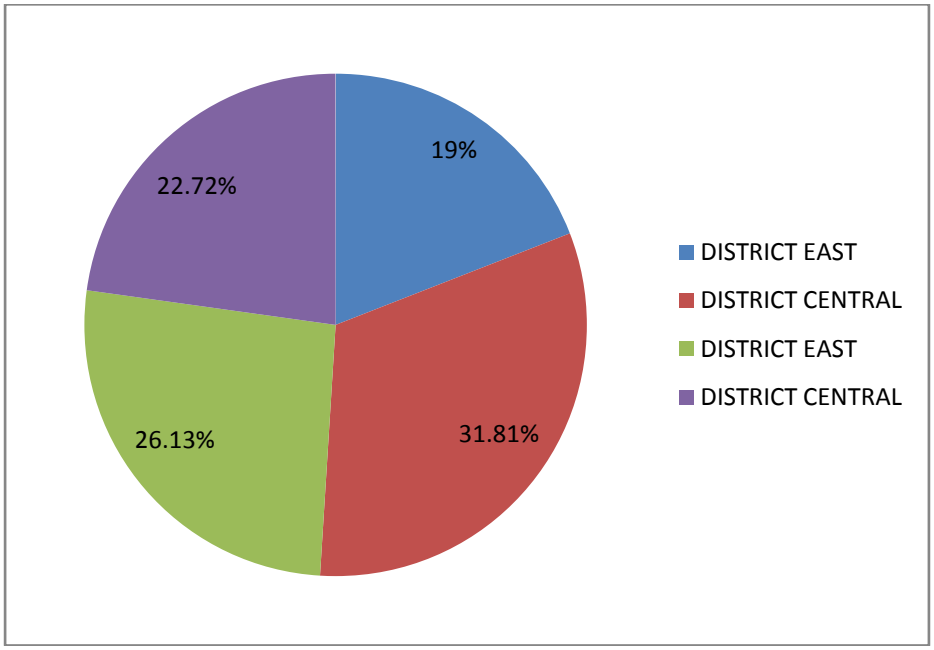

Figure 1 : Pie Chart Showing District Wise Collection of Serum and Blood Samples

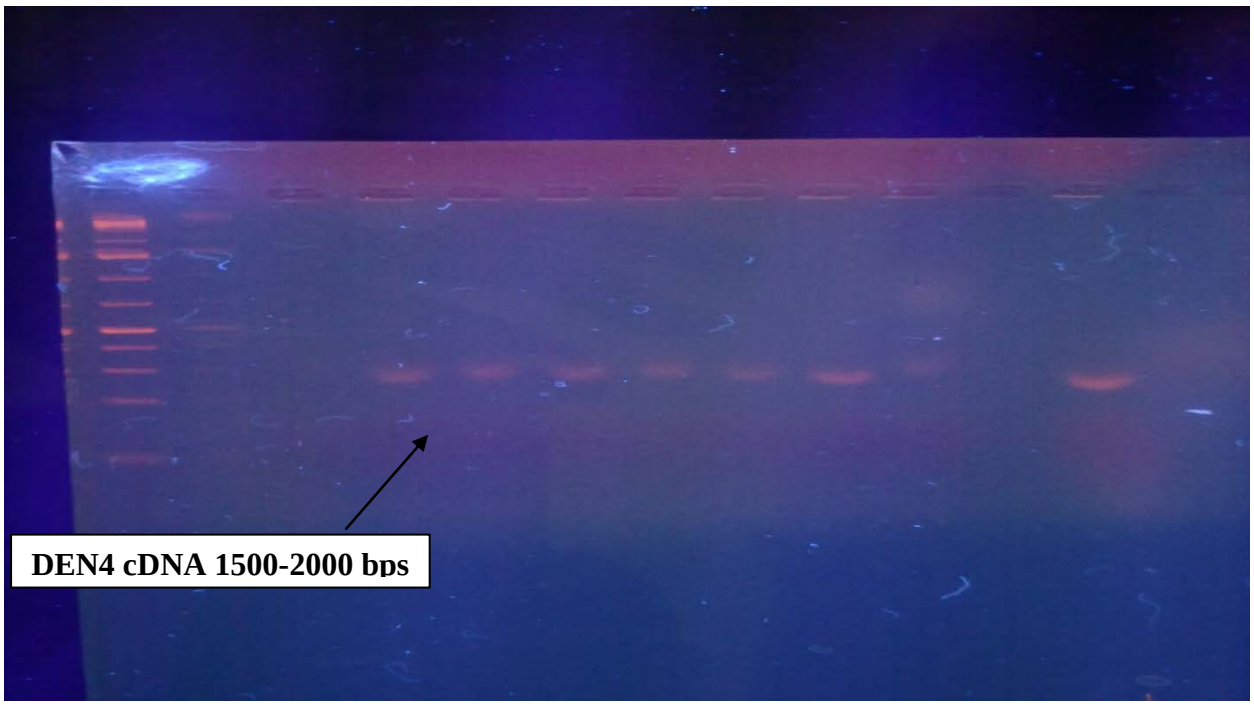

Figure 2 : Agarose gel electrophoresis shows DEN 4 cDNA bands 1,500-2,000 bps

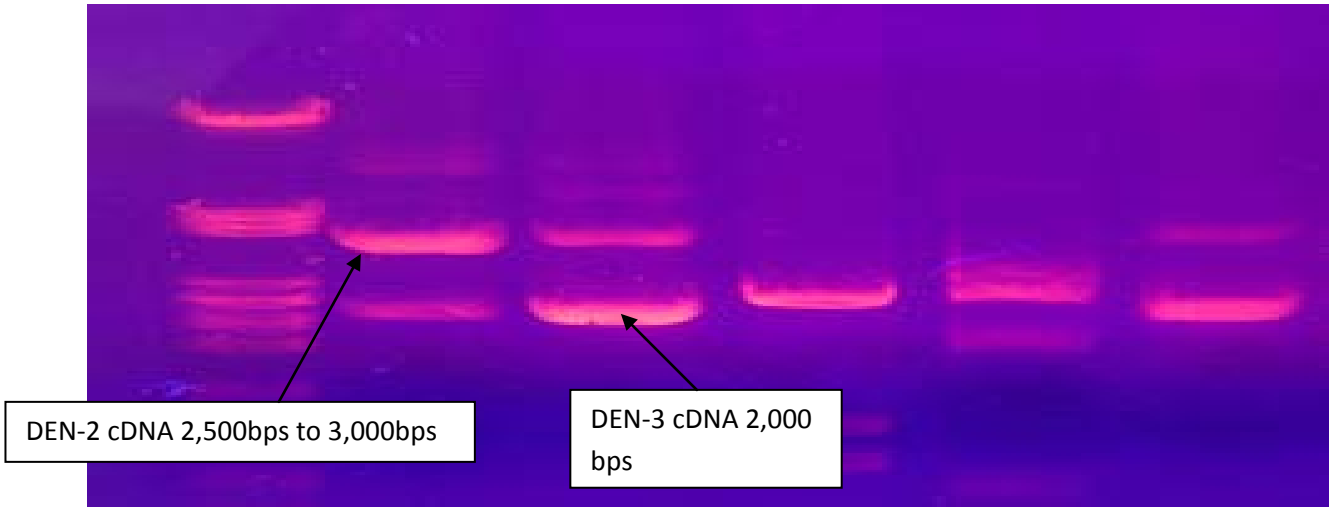

Figure 3 : Agarose gel electrophoresis showing DEN 2 and DEN 3 cDNA bands 2,500 bps-3,000 bps and 2,000 bps respectively