Immuno Flourescene Technique and One Step Real Time RT PCR Technique for Diagnosis and Eliminating Dengue Infection

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Abstract: *By getting the results of the study, it is clear that both the immune florescence technique and Real time RT PCR one step technique are effective and quick techniques for diagnosis and for research purpose and immune fluorescence technique is very beneficial if it is fully set in a local context and through these finding we also find using immune fluorescence technique, one step real time RT PCR and agarose gel electrophoresis that the dengue serotype 4 were the cause of major dengue infection in year 2012, and dengue serotype 2 and 3 were cause dengue infection in very small percentage. Dengue serotype 4 which is the geographically dominating serotype which is present in most areas of Karachi district, and dengue serotype 2 and Dengue serotype 3 were found almost everywhere in Karachi region. This is an threatening condition for the future, because it may be possible in the next monsoon season every 1-4 serotype dengue epidemic will be circulated and will happen which may causes more sever mix infection So before the monsoon season we should take steps to eliminate the infection and prevent dengue life of severe dengue infection. This can be helpful in eliminating the infection of dengue in the coming years.*
Keywords: *PCR, ReverseTtranscriptase RT-PCR, Immunofluorescence technique, Dengue fever, Dengue Virus.*

I. Introduction

The basic purpose of this research is rapid and quick identification of Dengue Virus for management of patient. Mosquito is the major vector of transmitting virus which is the causative agent of Dengue fever. There are four serotypes responsible for causing Dengue fever found named as: DEN-1, DEN-2, DEN-3 and DEN-4. Dengue fever is a Arbovirus disease that causes illness in humans. According to World Health Organization (WHO), the size of disease increased about thirty percent in the last 50 years Dengue virus serotype 1 to 4 is transmitted to human through mosquitoes named Stegomyia aegypti (formerly called Aedes aegypti) and many symptoms seems like disease (dengue) bleeding (hemorrhagic dengue fever) caused by type of dengue virus Flavivirridae of the family of flaviviruses. The single-stranded RNA of dengue virus encodes for three structural proteins called Envelope protein (E-Protein), Membrane protein(M-protein) and Core protein (C protein), RNA genomics produced the protein-rich poly precursor, a translation of these proteins done by virus-encoded proteases and producing individual host cell protein dengue virus NS1, NS2A, NS2B, NS3, NS4a, NS4b and NS5 (NS) non-structural proteins, and the RNA enclosed in a icosahedral capsid lipid bilayer with E-Protein, and M-protein through nucleocapsid. The dengue virus has diameter of nearly about 500 Å and spherical particle. A lipid envelope of about 10nm depth covers the nucleocapsid. The diameter of whole virus particle is about 50 nm and the density of viral particle is about 1.23 g/cm3.

The dengue disease classification has been developed by clinical professionals initially on the start of the practice among children in Thailand and the World Health Organization (WHO) published in 1975 and it restructured in 1997. The infection of dengue is ranked in the undifferentiated febrile illness, dengue fever (DF) and dengue hemorrhagic fever. WHO has contributed to assessment of the load of dengue infection worldwide and improved processing, due to which mortality rate of DHF become high. But the categorization scheme is developed on clinical symptoms of the disease and laboratory principles. Its ability to classify the pure dengue infection is not seriously related and submitted to this. So many studies have demonstrated many dengue cases and address strict DHF surely.

II. Materials & Methods

2.1 Data analysis procedure:
2.1.1. Immunofluorescence staining:

Immunofluorescence staining procedure was used for the identification and detection of viral antigen dengue which are structural proteins C (core protein), M (membrane protein) , and E (protein envelope) . To perform the immunofluorescence staining technique , we followed the protocol of Sirichan Chunhankan et al ; 2009 (316) according to him, first dilute the whole blood with a dilution buffer in the ratio of1 : 1, the dilution buffer was made by adding 10-15 Hanks balanced salt solution with 1 mM EDTA , 0 , 5 % bovine serum

albumin , ficoll and without calcium was then placed on the diluted blood and centrifuge at 250 g for 30 minutes at the interface was pooled and centrifuged at 1200 g for 5 minutes, then for 5 minutes washed with dilution buffer , and then coated was made on glass plate 100 ul PBMC pellet , atleast for 2 hours smear was air dried , and fixed and room temperature with 4% paraformaldehyde for 10 minutes, the loan smears stained for 1 hour with fluorescent isothiocyanate -conjugated polyvalent antiserum dengue 1-4 , then washed smear with phosphate buffered saline (pH 7.4) for 5 minutes, this washing operation with saline phosphate buffer was repeated three times, and then dried in air and examined under a fluorescence microscope at a magnification of 200X.

2.1.2. RNA isolation:

RNA was isolated fr om 85 serum samples from patients suspected of dengue , using the extraction kit (spinTM viral gene) in the intron.

In this protocol 150ul sample was collected in a tube with an Eppendorff which was added 250ul of lysis buffer, then vortexed for 15 sec , and incubated at room temperature for 10 minutes. After incubation at room temperature added 350ul of binding buffer and again vortex for 15 seconds. The sample was transferred to the column spinTM viral gene , which contains membrane silica column which retains RNA. This column was placed in the collection tube , centrifuge the sample containing column at 13 000 rpm for 1 min , and then changed the collection tube with a new one and 500 ul of wash buffer A , centrifuged again at 13 000 rpm for 1 min , then again added changed the collection tube with a new one . Then we added 500 ul of wash buffer in the column B to spin , centrifuged at 13,000 rpm for 1 min Discard the collection tube and placed in the 1.5 ml column of an RNase micro centrifuge tube connection. A 60 ul of elution buffer was added and incubated for 1 min and then centrifuged at 13 000 rpm for 1 minute, after the spin column discarded because tha RNA was eluted in the free micro centrifuge tube and RNase collect purified RNA . This RNA was purified subsequently subjected to multiplex Real Time RT PCR.

2.1.3 One step Multiplex Real Time RT PCR:

The isolated RNA was amplified by a step multiplex real- time RT-PCR. This technique is used to amplify the small amount of the RNA or makes copies of the RNA. The purified RNA was amplified using Ready -to -Go beads __gVirt_NP_NN_NNPS < __ Tag PURE PCR (Amershan Biosciences UK.) . These beads contain buffer , dNTPs , polymerase recombinant inbred Tag stabilizers and BSA and the system by which I performed in multiplex real-time RT-PCR thermal cycler is Cfx96TM Real Time C1000 Touch (Bio Rad) labeled with two dyes tell fam / vic . The four dengue virus primers were lyophilized , which were first diluted with TE buffer , then from this stock solution of the primer , we took a ul and added to TE buffer 9ul to make the job primary solution . From each primer working solution added 0.6ul and pure Tag -A- Go PCR Beads Ready pipe and this added 1 ul M-MLV Reverse Transcriptase (Promega) and 5 ul of reaction buffer M-5X MLV (Promega) and 7UL RNA isolated . The final volume of 25ul was made by diluting this mixture main reaction tube with extenders . Thermal cycle of dengue in real-time RT-PCR was created. Initially, the RNA was reverse transcribed to DNA c by providing the temperature of 55 ° C for 10 minutes, this was followed by a cycle of denaturation at 95 ° C for 8 min, following amplification was proceede with 50 cycles at 95 ° C for 10 s and a cycle at 60 ° C for 1373)

2.1.4. Agarose Gel Electrophoresis:

In electrophoresis on agarose gel I observed the size of the cDNA amplified using DNA marker (Lader) . In this technique of first 2.0gms agarose was taken and added to a 100 ml 1x TBE buffer and dissolved by gently heating in a microwave oven until transparent appearance . Cool slightly and add the agar 8ul bromide ethedium in it as a colorant. After adding dye pour the gel electrophoresis plate and PLAE comb it and wait 30 minutes for the solidification of the gel. After that when the gel is completely solidified , the gel has released the comb and pour 1000ml 1x TBE buffer in the electrophoresis chamber . Add loading buffer the sample and 2UL 10uL amplified cDNA into a small tube and mixed, then pipetted onto about 5-10ul and transfer it in an agarose gel of 2% in wells in a well added approximately 3UL d ' DNA ladder (fermanents 1kb Ready- to-Use) , attach the respective son to the room and adjust the voltage up to 75 volt current . For this reason cDNA increase from the negative pole to the positive pole of wait for two hours, after that when cDNA on an agarose gel at 2% , it will be placed in the hind gel unit (Alpha Innotech Fluorchem) for observation and photography. And observed cDNA bands and compared these cDNA bands with DNA ladder (fermantas 1kb Loan - ve) .

2.2 Data Collection Procedure:

There were eight serum samples from patients with suspected dengue, different hospitals and laboratories Karachi city during the months of August to November 2012.One 85 samples; 7 serum samples

were collected from the Dow University Health Sciences , 20 serum samples from laboratory Essa, 28 serum samples Imam Clinic and 20 serum samples were collected Memon Medical Institute , and 03 whole blood samples taken from patients positive for dengue (IgG and IgM positive) the same group of patients whose serum had been collected from medical Memon Institue as shown in Table 2 and figure 2 .Table 3 shows percentage of dengue sero-types.

Table # 2 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 institite (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%)

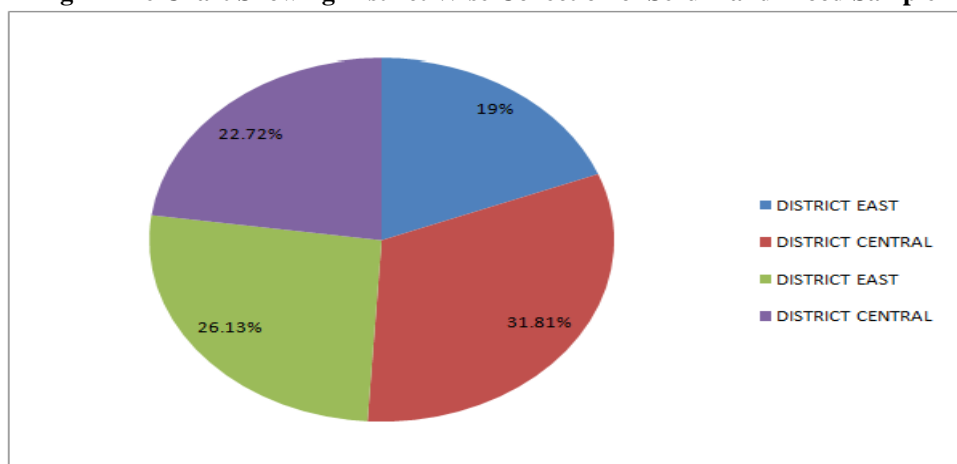**Fig # 2 Pie Chart Showing District Wise Collection of Serum and Blood Sample**

Table # 3 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%)

III. Results & Discussions

3.1 Result:

      The total of 85 serum samples were taken from patients suspected of dengue dofferent hospitals and laboratories Karachi from June to July 2014. On 85 serum samples 17 samples collected from Seru DOW Science University health , 20 serum samples from Essa laboratory and diagnostic center , 28 Imam clinical serum samples and 20 serum samples from medical institute Memon shown in Table 2. RNA was extracted from these putative serum samples using -spin viral gene (im) isolated RNA was subjected to a step multiplex real-time RT-PCR and then treated two percent agarose gel electrophoresis . After completing all the steps it was observed that all 85 serum samples from different patients suspected dengue rtPCR positive results in real time and also produce related bands on agarose gel . The strips of 2 percent agarose electrophoresis gel dengue cDNA sizes give approximately equal lengths or which are of the order of 1500 bps to 2000 bps , and some are of the order of 25,000 bps at 3000 bps.

      The molecular weight of isolated bands were in the ranges from 1500 bps to 2000 bps and 25,000 bps to 3000 also mentioned by other scientists bps and confirms the accurate dengue virus . It is important to note that the majority of the isolates were dengue type 4 to about 84 %, while dengue serotype 2 is 10 % and type 3 dengue sero is only 6% showed in Table 3 the total three whole blood samples positive (IgG and IgM) in serum dengue patients were collected and preceded comes immunoflorescense staining it was the first time we had time to use immunofloresense staining for the detection of the virus in Karachi, Pakistan . We must try to establish each requirement and all necessary equipment . That is why we tried first with only three 100% of the positive samples and the results have been promising our finding suggests that DENV 2 - infected mobnocytes were present in the slides as those observed using immunoflorecsense microscopi and shown green fluorescent objects.

3.2 Discussion:

Molecular techniques are very useful and beneficial in a direction of the detection, identification and typing of dengue virus and epidemiological studies also . Initially in this study , I tried to establish immune fluorescent technology in the local context , but due to the unavailability of the necessary reagents and equipment, we had tried this technique on three samples of whole blood and get positive results gradually for DEN- 2 , this means that fluorescent immunoassay technology is a simple and fast technique if we establish it in the local media , however , due to the low socio-economic conditions that we tried to establish this technology in a frame local . And in this study we detect also identify and evaluate this, which dengue serotype circulating in our community or environment to this end with immunofluorescence technique we have also stepped multiple real-time RT-PCR. Total serum samples of 85 suspected dengue patients were collected from different laboratories and hospitals in Karachi. Isolated RNA was subjected to real -time multiplex RT-PCR step and 2% agarose respectively . After performing a real-time multiplex RT PCR step , we obtained positive results mean that all serum samples of 85 dengue patients had suspected dengue RNA, the majority of 85 patients suspected of dengue had length 2% agarose which is of the order of 1500 bps to 2000 bps and very few bands are in range of 2500 bps to 3000 bps, it also suggests that the majority of the 85 serum samples from patients suspected dengue almost shared the same serotypes in Karachi city in the year climate in 2012.

Anna P. Durbin et al , 0.2001 (374) indicated that the size of the serotype of dengue virus 4 complete genome is 2000 bps in another study Juh Lai Ching et al (375) showed that the size the complete genome of the serotype of dengue virus is 1343 bps , it means that the size of the complete genome of serotype 4 dengue virus is in the range of 1 to 2 bps ooo , ooo bps while the size of the virus dengue serotype 2 by Stephanie polo et al , 997 (376) is 3.2 kb which is about 3200 bps, Lewis Markoff et al, 2002 (377) , said that the size of the serotype of dengue 1 is 1 to 1.5 kb , which is about 1000 to 1500 bps. According to Joseph E. Blaney JR, et al , 2004 (378) dengue serotype 3 was about 2000 bps , but according to our results, we have observed on agarose gel 2% to about 84% of the bands were of order of 1500 bps to 2000 bps, and dengue serotype 2 was present in about 9% and the dengue serotype 3 was present in about 6% of this , it means that the dengue serotype 44, which is of approximately 84 % was the main cause of outbreaks of dengue in Karachi in 2012. Erum Khan et al, 2010 (144) in the dengue serotype 2 2004 year was dominant and the year 2005, serotype of dengue virus 3 dominated . According to Muhammad Idrees et al , 2012 (3700) for the year 2006-2009 for both dengue serotype 2 and serotype 3 dengue were so circulated in 2011 and dengue serotype 2 was dominant . Our results showed that in 2012 dengue serotype 4 was more dominant than dengue serotype 2 and serotype dengue 3 and may be possible to cause several dengue infections -to say , dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) during that year . Situation in the coming years particularly for patients who had previously infected with dengue infection and secondary infection with one of four serotypes of dengue the resultant will be more severe this is why it is recommended take serious steps to eliminate dengue infection our region and make every effort to warn people against infection of dengue.

Secondly, a multiplex Real Time RT PCR step detected viral RNA dengue and dengue viral RNA reverse transcribed into complementary DNA (Edna) , the evidence showed that all 85 serum samples from suspected dengue patients were collected early phase , it is because the RNA remains in human blood for only 5-6 days of early infection, after immunnglobulin M (IgM) produced in the blood and begin to remove the dengue virus , after which the immunoglobulin G (IgG) product, and also the elimination of dengue virus and IgG remains in the blood for long periods of time and to grant immunity to those same stereotype . That's why all 85 serum samples were diagnosed early phase serum samples and had shared almost serotype of dengue virus in Karachi city in 2012. This result helps in the epidemiological study and assistance in data collection for monitoring infection with dengue virus in Karachi city.

Karachi city is geographically distributed in five districts which are ; Central, South , East, West and Malir district. As we collected samples from different hospitals and laboratories Karachi city which is located in different parts of Karachi city, the largest number of samples of dengue were received from central neighborhood that are about 48 in number and number lower dengue samples were received in the district of which is about 40 in number , so in this study, we also find that in 2012 dengue serotype 4 was distributed in the central area of the is Karachi city while other dengue serotypes which are dengue serotype and dengue serotype 2 March .

IV.   Conclusion

The results of this study shows that both real-time and an immunofluorescence step RT-PCR technique is effective and rapid techniques for diagnostic and research purposes and immune fluorescence technique is very beneficial if it is fully set in a local and long under these finding we also find using immune fluorescence technique , a real-time RT-PCR step and agarose gel electrophoresis the main dengue virus infections in 2012 were dengue serotype 4 , while dengue serotype 2 and serotype 3 dengue were present in very low percentage . Geographically dominant dengue serotype 4 was present in most of the city districts of Karachi while , dengue

serotype 2 and 3 were also present in almost all districts of Karachi city. This is an alarming situation for the future, because it may be possible in the next monsoon season every 1-4 serotype dengue epidemic will be circulated and will happen which can cause co-infections with more severity so before the monsoon season we should take some measures for the elimination of dengue infection and prevents the life of severe dengue infection. This can be helpful in eliminating the infection of dengue in the coming years.

4.1 Future Aspects:

Our study will provide a very beneficial immunofluorescence technique for the diagnosis and research purpose of this technique is very quick and fast if others in the future will be in place in a local context , because we do not have pretty regent and equipment for technical immunofluorescence and that's why we had tried this technique on three blood samples and obtain progressive results , which is why we have said that this is the technique is very useful to future for further epidemiological study and our study will be useful in all the illumination of infection with dengue virus in our community through different strategies and different management of dengue infection and it can useful in the future to work on the development of a vaccine.

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