DEVELOPMENT OF A NOVEL-UNIVERSAL MULTIPLEX RT-PCR KIT FOR RAPID DETECTION OF VECTOR-BORNE VIRUSES

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ABSTRACT

The study was designed to develop a rapid and simultaneous detection of most important arbo-viruses of human being of the tropical and sub-tropical countries of the world. ELISA and other serological tests, RT-LAMP, NASBA and conventional RT-PCR are being used for the detection of antigens and antibodies of Dengue(1-4),Chikungunya, Japaneseencephalitis and West Nile viruses all over the world. All these tests are time consuming, laborious and costly and the result of diagnosis is some times confusing. Any of the above mentioned tests which is being designed for the detection of one virus genome other viral genome remains undetected. The sensitivity and specificity of a multiplex RT-PCR depends on the design of primers, uses of enzymes and condition of nucleic acid amplification. In this study, three DNA-polymerase (LA-Taq, r-Taq and Tth) and two reverse-transcriptase (AMV-RT and RT-ACE) were used. Highly gene specific primer was designed against each of the four sero-types of Dengue and other sero-types of Chikungunya, Japaneseencephalitis and West Nile viruses to increase the sensitivity and specificity of MRT-PCR. Of the six combinations, AMV-RT (Reverse Transcriptase) and La-Taq (DNA Polymerase) combination was found the best in terms of sensitivity and specificity of the MRT-PCR, which could detect minimum number of any of the four species of arbo-viruses obtained either from the clinical or laboratory or field samples. The test was found cost effective (TK 50.0 BD/ UD$ 0.60) , rapid (1.5 hours) and sensitive (0.1 FFU can be detected). Anyone, either from the developed or developing countries having minimum knowledge on PCR can easily performs the test either in the hospital or in a diagnostic center dealing with bulk samples where the diseases are endemic.

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INTRODUCTION

A large number of viruses of the diverse family *Flaviviridae* and *Togaviridae* infect both arthropods and vertebrates. Member viruses of these families that infect humans frequently cause severe morbidity and mortality, their epidemic continues to be a major public health threat all over the world. The medically important viruses of the families' *Flaviviridae* and *Togaviridae* are distributed all over the world, i.e. four sero-types of dengue virus (DEN 1-4) distributed in the most tropical and sub-tropical countries of the world; Chikungunya virus (CHIK) and rose river virus (RRV) in Africa, Asia and Australia; Japaneseencephalitis virus (JEV) in Asia and Australia; West Nile virus (WNV) in Africa, Central Europe, and recently in North America; yellow fever virus (YFV) in Africa and Latin and South America; tick-borne encephalitis virus (TBEV) in the temperate region of Europe, North America and Asia [(1)]. Dengue is considered as the tenth leading cause of human death in the world. The disease is caused by any sero-types of dengue, an enveloped single stranded positive sense RNA virus. All most a half of the world's population is at risk of being infected with the members of the genus flavivirus, with 2.5 billion people at risk of infection from dengue alone [(2-5)]. Most mosquito borne viral encephalitis of human and animal are caused by any member viruses of the family *Flaviviridae*, i.e. JEV, WNV / Kunjin virus (KJV), St. Louise encephalitis virus (SLEV) and TBEV. All the member viruses are equally potent to develop severe form of encephalitis both for man and animal and leads to the death of the patient [(6)]. A viral fever manifesting similar symptoms of classical dengue fever caused by CHIK, a single sero-type, positive sense RNA virus of the family *Togaviridae*, transmitted by the same mosquito vectors of dengue.

At present, three methods are commonly used for the diagnosis of *Flaviviridae* and *Togaviridae* infection by virus isolation and characterization, detection of genomic sequence by nucleic acid amplification technology and detection of virus specific antibodies [(7- 11)]. Several serological tests and methods have been designed for the diagnosis of the diseases caused by the member viruses of the family *Flaviviridae* and *Togaviridae* i.e. HI, SNT, PRNT, IFA, IgG and IgM capture ELISA [(12- 20)]. Of all the serological tests, the IgM and IgG ELISA are widely used for rapid serological diagnosis, using a single or paired serum sample, which also do not furnish information about the sero-type determination of the viruses [(18-20).] Serologically it is very difficult to differentiate one member virus of *Flaviviridae* responsible for encephalitis from other due to cross-antibody reaction [(16)]. The serodiagnosis of the past and present dengue, JEV, WNV and CHIK viruses' infection is difficult due to the long persistence (≥10 months) of IgG antibodies against them and rather complicated for various reasons.

A single-step reverse transcriptase PCR (RT-PCR) detection and typing of dengue and other viruses offers a sensitive, specific and rapid alternative that requires only one acute-phase serum sample. This technique can be made cost-effective by following low-cost methodology, like development of multiplex reverse transcriptase-PCR (MRT-PCR) [(21-23)]. To reduce time and minimize complications, the two-step approach was later modified to a single-step multiplex RT-

PCR system for the genome detection and typing of dengue viruses. However, the multiplex RT-PCR system designed [23] for detection and typing of dengue viruses only was found less sensitive. More sensitive, specific, rapid, reliable and economic single-tube-single-step multiplex RT-PCR are therefore needed to complement the existing PCR-based assay systems for the detection of more than one viruses both from the clinical, laboratory and field samples.

The present study describes the development and evaluation of a highly sensitive, specific, rapid, cost-effective and a simple, one-step-one-tube MRT-PCR for the rapid detection, serotyping of DEN and differentiation of dengue from CHIK viruses and JEV from WNV where these diseases are endemic. Therefore, the results on the sensitivity and specificity of the MRT-PCR technique are reported, the applicability and feasibility of use of the technology for the clinical, laboratory and mosquitoes samples in the endemic areas are discussed in this study.

MATERIALS AND METHODS

HUMAN PATIENT SERUM SAMPLES. The serum samples used in this study were collected from confirmed DEN or clinically suspected DEN or CHIK infection from Bangladesh of the outbreak year (2002), Thailand (2003-2004) and Indonesia (2003-2004).

RNA EXTRACTION. The viral RNA was extracted from 140 µl of infected culture fluid (ICF) and spiked mosquito homogenate (SMH) with known focus forming unit (FFU) of virus and 70 µl of patient serum samples using the QIAamp viral RNA mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The RNA was eluted from the QIAspin columns in a final volume of 60 µl elution buffer and used for RT-PCR immediately.

RT-PCR REACTION MIXTURE. For 50.0 µl uniplex RT-PCR reaction volume contained a mixture of 10 X LA PCR buffer (5.0 µl), 2.5 mM $MgCl_2$ (4.0 µl),10 mM dNTP Mix (2.0 µl), Prime RNase Inhibitor (2.0 µl), LA *Taq* (0.2µl /2 units), AMV-RT (0.2 µl/ 2 units), type specific primers (sense primer 0.15 µl (15 nmol) to 0.4 µl (40 nmol), RNA template 1µl to 4µl depending on virus titer (100 - 0.1 FFU) and DEPC treated water (33.25 to 30.0 µl)

*MRT-PCR REACTION MIXTURE***.** For a 50.0 µl reaction volume of both the mRT-PCR , contained a mixture of 10 X LA PCR buffer (5.0 µl), 2.5mM MgCl2 (4.0 µl), 10mM dNTP Mix (2.0 µl), Prime RNase Inhibitor (2.0 µl), LA *Taq* (0.2 µl /2 units), AMV-RT (0.2 µl/ 2 units), primers, dengue consensus sense primer DCS1, 0.15 µl (15 nmol) and type specific complementary primer for each dengue sero-type, D1C, 0.2 µl (20 nmol); D2C, 0.15 µl (15 nmol), D3C, 0.15 µl (15 nmol), D4C, 0.20 µl (20 nmol), CHIK-S, 0.4 µl (40 nmol) ,CHIK-C, 0.4 µl (40 nmol), JEV-S, 0.4 µl (40 nmol), JEV-C, 0.4 µl (40 nmol), WNV-S, 0.2 µl (20 nmol) and WNV-C, 0.2 µl (20 nmol) and RNA template 2 µl for each virus (titer 100.0-0.1 FFU) for each MRT-PCR reactions and DEPC water (33.25 to 30.0µl), respectively.

SENSITIVITY AND SPECIFICITY OF THE MRT-PCR WITH THE SPIKED VIRUSES. The sensitivity and specificity of mRT-PCR assay for the detection of DEN, CHIK, JEV, WNV specific RNA in the mosquitoes specimens were evaluated with *Aedes aegypti* and *Culex tritorincus* mosquitoes homogenates spiked with known FFU (100 to 0.1) of the above mentioned viruses in the absence of real or field collected mosquitoes specimens. Prior spiking, laboratory reared mosquitoes homogenate were screened by RT-PCR and MRT-PCR using specific sets of primers. Following spiking, all of the samples were processed for RNA extraction with a QIAamp viral mini kit (QIAGEN) and screened by MRT-PCR for detection of the above mentioned viral RNA.

REFERENCE VIRUS STRAINS. The virus sero-types and strains used in this study were dengue virus sero-type-1 (DEN 1/Thai strain, DEN1/Hawaii), dengue virus serotype-2 (DEN 2/ ThNh7/93, DEN 2/SL-04-111, DEN 2/oost-22A), dengue virus serotype-3 (DEN 3/BDH02-02, DEN 3/SL-03-11701, DEN 3/SLMC-50), dengue virus serotype-4 (DEN 4/SL-04-8532, DEN4/SLMC-318, DEN4/17), Chikungunya virus (CHIK; S27 African prototype), CHIK/M29-98, CHIK/M16-98), Japaneseencephalitis virus (JEV/JaOArS982, JEV/Strain 0566, JEV/JaNAr02-90, JEV/JaNAr05-90) and West Nile virus (WNV/ NY-99, WNV/ Eg-101).

RESULTS

PRIMERS SPECIFICITY FOR RT-PCR AND MRT-PCR ASSAYS. To establish a highly sensitive uRT-PCR followed by mRT-PCR for the rapid genome detection of viruses of the family *Flaviviridae* and *Togaviridae* used in this study. The newly designed primer sets for MRT-PCR I revealed higher degree of specificity and sensitivity for the detection of viruses from the serum, ICF and MH with the LA *Taq* DNA polymerase and AMV-RT enzymes combination, compared to the other five combinations. Among the five different combinations of two-enzyme protocol, although the sensitivity and specificity of the enzymes combination of the *rTaq* and AMV-RT was found relatively better in the genome detection of all the four dengue sero-types and CHIK than the enzymes combinations of the *Tth* and AMV-RT for the uRT-PCR, but none of the combinations of the two enzymes protocol, *rTaq* and AMV-RT and *Tth* and AMV-RT revealed equal sensitivity for the MRT-PCR despite maintaining similar condition of each PCR assays in this study (Fig.1,2,3,4,5).

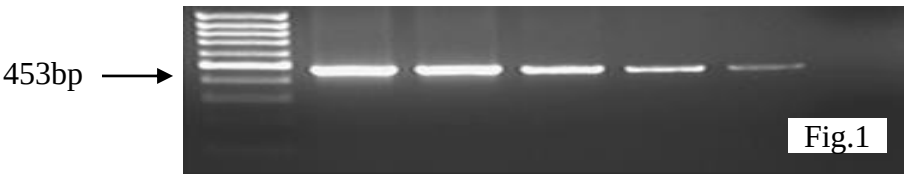

Fig. 1. Electrophoresis of PCR products on 2% Agarose NA showing the sensitivity of MRT-PCR/LA-Taq+AMVRT. Left margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 indicating 100 FFU of spike DNA. Lane 2 fifty, Lane 3 twenty, Lane 4, 10 and Lane 5, 1 FFU of Dengue 1 viruses

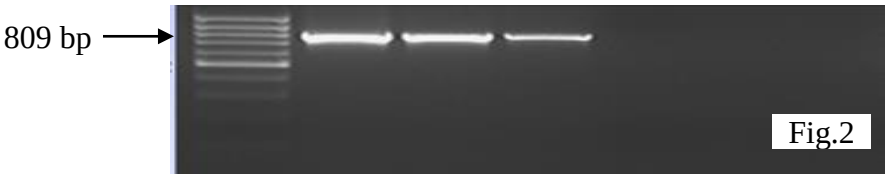

Fig. 2. Electrophoresis of PCR products on 2% Agarose NA showing the sensitivity of MRT-PCR/LA-Taq+AMVRT. Left margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 indicating 100 FFU of spike DNA. Lane 2 fifty, Lane 3 twenty FFU of Dengue 2 viruses.

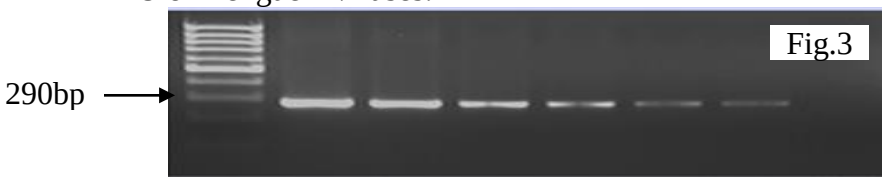

Fig. 3. Electrophoresis of PCR products on 2% Agarose NA showing the sensitivity of MRT-PCR/LA-Taq+AMVRT. Left margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 indicating 100 FFU of spike DNA. Lane 2 fifty, Lane 3 twenty, Lane 4, 10, Lane 5, 1 and Lane 6, 0.1 FFU of Dengue 3 viruses.

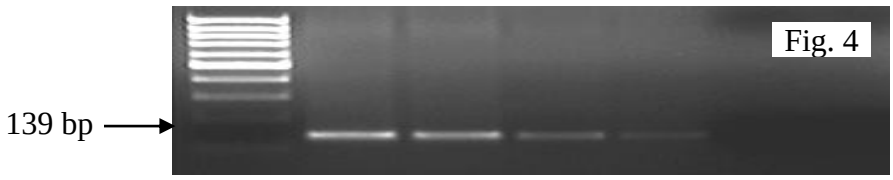

Fig. 4. Electrophoresis of PCR products on 2% Agarose NA showing the sensitivity of MRT-PCR/LA-Taq+AMVRT. Left margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 indicating 100 FFU of spike DNA. Lane 2 fifty, Lane 3 twenty and Lane 4, 10 FFU of Dengue 4 viruses.

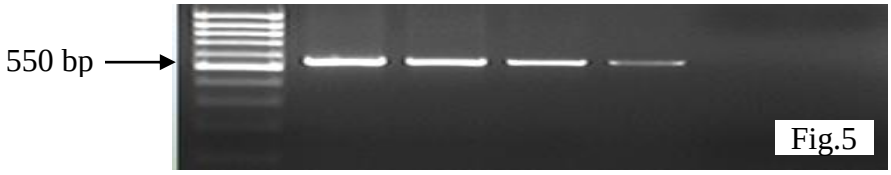

Fig. 5. Electrophoresis of PCR products on 2% Agarose NA showing the sensitivity of MRT-PCR/LA-Taq+AMVRT. Left margin of the gel indicating 100 bp DNA marker

(Invitrogen, USA). Lane 1 indicating 100 FFU, Lane 2 fifty, Lane 3 twenty and Lane 4, 10 FFU of Chikungunya viruses.

SENSITIVITY AND SPECIFICITY OF THE MRT-PCR ASSAYS. The similar primer-pairs used for the rapid detection and differentiation of the individual viruses by RT-PCR, were selected for the MRT-PCR in this study. The primer-pairs designed for the RT-PCR revealed equal sensitivity and specificity for the single-tube-single-setp MRT-PCR assays too. The limit of genome detection by MRT-PCR were found similar to that of RT-PCR used in this study. On the contrary, only 19, out of 317 serum samples were positive by virus isolation (shown in Table 1). The sensitivity and specificity of the MRT-PCR used in this study were found higher in the case of the two-enzymes combination protocol of LA *Taq* DNA polymerase and AMV-RT compared to those of other five combination .

Table 1. Evaluation in sensitivity among RT-PCR, MRT-PCR and isolation of viruses from acute phase dengue suspected patients

Countries	**Type & number of sample (Serum)**	**RT-PCR Positive**					**MRT-PCR positive**					**Virus Isolation positive**				
		D1	D2	D3	D4	Total	D1	D2	D3	D4	Total	D1	D2	D3	D4	Total
Bangladesh	100	0	0	12	0	12	0	0	14	0	14	0	0	6	0	6
Thailand	84	2	6	3	3	14	2	8	4	3	17	1	2	2	1	6
Indonesia	133	1	1	3	8	13	1	1	5	11	18	0	1	2	0	3

D1 indicating Dengue virus type 1, D2 indicating Dengue virus type 2, D3 indicating Dengue virus type 3, D4 indicating Dengue virus type 4.

EVALUATION OF MRT-PCR I & II SENSITIVITY WITH THE SPIKED VIRUSES. The applicability of the MRT-PCR I & II for the genome detection and differentiation of the viruses spiked with vector mosquitoes homogenate, and the results were compared with the RT-PCR. A known FFU (100-0.1) of each of the four viruses (DEN 1-4, CHIK, JEV and WNV) were spiked

with 10 times higher RNA of the specific vector mosquitoes (*Aedes aegypti*, *Culex tritorinchus*) cell homogenate. Each of the spiked viral RNA template was used for MRT-PCR and RT-PCR respectively (Fig.6,7).

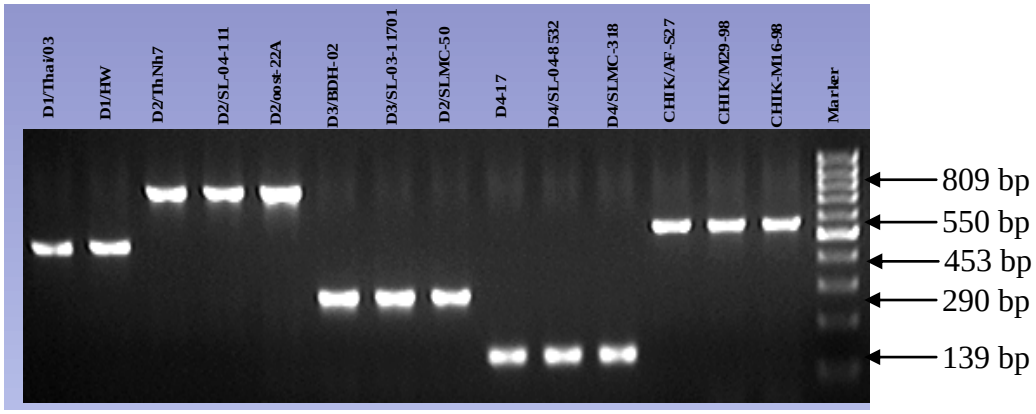

Fig 6. Electrophoresis of PCR products on 2% Agarose NA. Right margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 & 2 indicating dengue 1, Lane 3 to 5 indicating dengue 2, Lane 6 to 8 indicating dengue 3, Lane 9 to 11 indicating dengue 4, Lane 12 to 14 indicating chikungunya viruses.

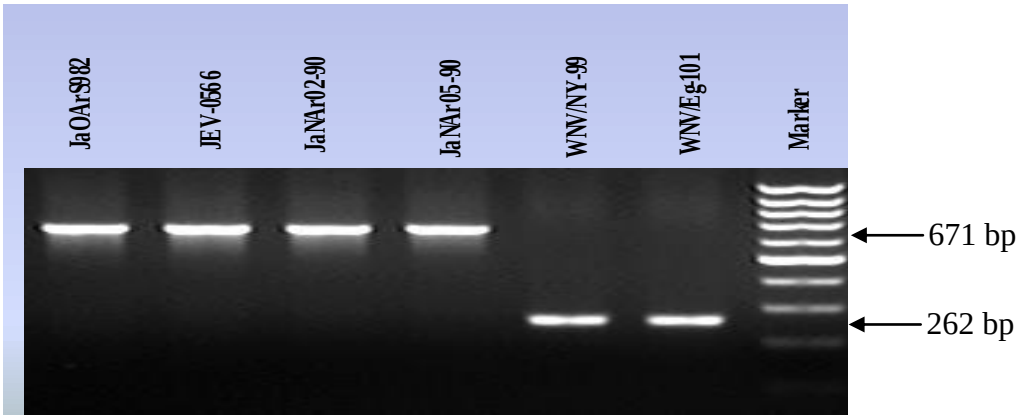

Fig 7. Electrophoresis of PCR products on 2% Agarose NA. Right margin of the gel indicating 100 bp DNA marker (Invitrogen, USA). Lane 1 to 4 indicating Japanese encephalitis virus Lane 5 and 6 indicating West Nile viruses.

The same primer sets designed for the MRT-PCR used in the RT-PCR for genome detection of each virus used. The sensitivity and specificity of the MRT-PCR used for the genome amplification of the template RNA of the spiked viruses was found remain identical with the RNA of the non-spiked viruses. The specificity of each primer set of the MRT-PCR was confirmed by using mosquito cell derived RNA as a control. Both the PCR systems were repeated three times for each isolate of the viruses to confirm a reproducible result on the sensitivity and specificity of the primer sets designed for the MRT-PCR in this study.

DISCUSSION

All the above-mentioned diseases are caused by the viruses of the family *Flaviviridae* and is transmitted by their specific vector mosquitoes (*Aedes aegypti/ Aedes albopictus* or *Culex spp*.) to man and animal. Vaccines are not available against any of the above mentioned deadly diseases except Japanese encephalitis. Although there is much more advancement in the development of vaccines against the dengue fever, Chikungunya viral fever and West Nile fever in the laboratory, effective vaccines against any of these three deadly diseases are not yet available in the market. A large number of populations of most tropical and subtropical countries of the world are in threat from these three diseases. Therefore, early diagnosis of these diseases either by antibody or nucleic acid detection or by virus isolation techniques are being practiced all most every countries in the world where the diseases are endemic or epidemic. Considering all these facts, early confirmation and rapid diagnosis of these diseases has been given more emphasis now a days in every endemic and epidemic countries. Therefore, during the past decade, various nucleic acid detection techniques, such as RT-PCR, two-step nested RT-PCR, real-time PCR and NASBA systems have been developed to address the need for the rapid detection and differentiation of the vector-borne viruses with more accuracy other than antibody detection and virus isolation [24- 30]. Although there are some reports on identification of the four dengue sero-types by nested amplification of a primary product generated with universal dengue primers by hybridization of a universal RT-PCR product with type-specific probes [31], by simultaneous amplification with four sets of type-specific primers or by use of a single 5' universal primer and four type-specific 3' primers. However, the performance of multistep nested amplification always increases the risk of cross-contamination, especially during routine diagnosis. Although the magnitude of nucleic acid amplification can be obtained by any of the above-mentioned PCR methods but none of them is suitable to detect more than one target gene of several viruses in a single-tube-single-step reaction. In addition, these methods are often cumbersome to be adapted for routine diagnosis both in the urban and rural areas.

In this regard, the RT-PCR assays reported in this study can be excepted as one of the sensitive diagnostic methods due to its simple operation, rapid detection, and high specificity and economically feasible. The newly developed MRT-PCR have a novelty to detect and differentiate all the four dengue sero-types from CHIK and also JEV from WNV rapidly from the clinical, laboratory and mosquitoes specimens without showing artifact in the results of diagnosis. There

are several reports of type-specific and group-specific RT-PCR and MRT-PCR for the detection of dengue only [22- 26]. The group-specific MRT-PCR that was developed first [21] for the detection and differentiation of the four sero-types of DEN by a single-tube reaction. Its sensitivity and specificity was found relatively less than the type-specific RT-PCR. Based on their idea we have developed these two MRT-PCR for the rapid detection of the four sero-types of DEN and differentiation of DEN from CHIK and JEV from WNV by single-tube-single-step reaction. To increase the sensitivity and specificity of our MRT-PCR we had to bring several changes in the system. Since our primary aim was to develop such a MRT-PCR which could be able to detect the genome of all the four DEN sero-types and CHIK virus in a single-tube reaction, we had to design a pair of sense and complementary primers for the CHIK virus. For that we had to replace two complementary primers for the DEN-2 and DEN-4 from the primer set used in the MRT-PCR . When the primer-pair of CHIK was added with the primer-set of the MRT-PCR described [23], more than three non-specific bands were appeared along with the specific band in the DEN-2 and DEN-4 PCR products of the MRT-PCR (data not shown). The amplification cycles of the two-enzyme system were also reduced from 40 cycles[31] to 25 cycles with a view to get more specific PCR products of each of the five viruses in a minimum time as well. In our study, we also noticed that changing of primers set, amplification cycles and temperature at the reverse transcription step were not enough unless and until we used the right combination and concentration of the two enzymes (DNA polymerase and reverse transcriptase). Of the six different combinations of the two-enzyme protocol system MRT-PCR, the enzymes combination, LA *Taq* DNA polymerase and AMV-RT was found the best combination compared to the other five combinations used in this study . Therefore, replacement of these two complementary primers (DEN-2 and DEN-4) of the previous primer-set, reduction of PCR amplification cycles, an increase of temperature at the reverse transcription step and the enzyme combination might have played tremendous role in the increase of the specificity and sensitivity of both the MRT-PCR in this study. A remarkable variation was noticed in the results of sensitivity and specificity of the MRT-PCR of the present study used for the detection and differentiation of the four DEN sero-types and CHIK compared to the MRT-PCR of (23,31) used for the detection of DEN four sero-types only. The possible reasons for the reduced sensitivity and specificity of the previous two mRT-PCR designed for the rapid genome detection of dengue viruses, might be, the use of higher concentration of primers (0.5-1.0 μM) for each of the four dengue sero-types, use of low temperature (42 ^{0}C) at the reverse transcription stage and the increase of PCR amplification cycles (40 cycles). Of all the abovementioned important factors those are considered to be responsible for the increase or decrease the sensitivity and specificity of the MRT-PCR, primers concentration can be given first priority then designing of primers. Like clinical and laboratory specimens, an equally potent RNA extraction method was required for proper detection of the viruses either from the infected vector mosquitoes or from the vector mosquito homogenates spiked specimen without RNA degradation or PCR inhibition. The RNA extraction efficiency of the QIAGEN kit from the spiked viruses used in this study indicated that this method could be used successfully for the extraction of RNA

without contamination from the viruses infected vector mosquitoes collected from the field as well. The RT-PCR and MRT-PCR conducted with the RNA extracted by the QIAamp mini extraction kit exhibited excellent sensitivity and showed no evidence of inhibition in amplification [24].

The sensitivity and specificity of the single-tube-single-step MRT-PCR were verified by using reference viruses of all the four sero-types of dengue, CHIK, JEV and WNV of different continents in this study, which indicated that these two molecular methods could be used confidently for rapid detection of the above mentioned vector-borne viruses.

At present, as there is no effective vaccines or any specific treatment available for dengue fever, chikungunya viral fever and West Nile fever, and the prospects of receiving the recent trend of increased epidemic activity and geographic expansion of all most all the important vector-borne diseases we discussed herein are not very promising. Moreover, presence of all the four-dengue serotypes has dramatically increased the chances of heterotypic infection, an important risk factor for the development of DHF and DSS. Therefore, in this scenario, the newly developed single-tube-single-step MRT-PCR based assays would prove to be very useful tool for the routine diagnosis of these four deadly viral disease of human and animal as a rapid, sensitive, specific and cost-effective assay both in the laboratory as well as in the field where these diseases are endemic. Even with several advantages, MRT-PCR with a single-pair of primers thus can detect only one target virus and is potentially expensive.

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