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Simultaneous detection of *Dengue* virus serotypes by nested-multiplex PCR

David Shayan[1,2], Reza Heidari[3] and Parviz Shayan[4,1]

[1] Institute of Molecular Biological System Transfer, Tehran, Iran
[2] Department of Biology, Kish International Campus, University of Tehran, Tehran, Iran
[3] Medical Biotechnology Research Center, AJA University of Medical Sciences, Tehran, Iran
[4] Research Center of Ticks and Tick-borne Diseases, Department of Parasitology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

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Abstract

Dengue virus is a single positive RNA virus causing *Dengue* fever (break-bone fever), belonging to the *Flavivirus* in the family of *Flaviviridae*. Four antigenically distinct serotypes have been reported from this virus developing infection with asymptomatic or clinical signs of high fever, headache, body aches, nausea and rash in human. This virus is mainly transmitted by mosquitoes *Aedes aegypti* or *Aedes albopictus* and in the last decade it has gained importance in the public health in Iran. It was reported that non-human primates, bovids, horses, pigs, birds, and rodents can be infected with this virus. Therefore, early detection of the virus in mosquitoes and humans can be important in controlling the disease. In the present study, we introduce a primer pair detecting *Dengue* virus (all four serotypes) and a nested-Multiplex PCR detecting each serotype simultaneously. For this aim we used recombinant plasmid constructs (D1-, D2-, D3- and D4- pEX-A128 plasmid) having ca. 660 nucleotides from 5` end of the corresponding serotype and larvae of *Aedes* mosquito. Our results confirmed that the 5` end of the virus is suitable for simultaneously detection of *Dengue* virus serotypes by RT-PCR and discrimination of serotypes from each other by multiplex PCR. In conclusion, we propose that to lower the cost, the *Dengue* virus should be preferably detected first by serotype common RT-PCR. In the case of positive result, the serotype determination can be performed by nested-multiplex PCR. Since for the management of public health, monitoring of the circulating *Dengue* virus is of great importance, the presented method is a suitable assay for this aim.

Introduction

Dengue (break-bone fever) is a systemic viral infection with often inapparent clinical signs but by some patients can lead to the clinical manifestation. This clinical manifestation can vary between mild to server disease (1). The causative agent of break-bone fever is *D*engue virus belonging to genus *Flavivirus* in the family *Flaviviridae* with four serotypes (DEN-1, DEN-2, DEN-3 and DEN-4) (2). Gubler (2006) described dengue as an old disease,

***Corresponding author:** *pshayan@ut.ac.ir*
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introduced first as clinically compatible disease in year 992 in china (3). But the virus was first isolated in 1943 in Nagasaki, Japan by Ren Kimura and Susumu Hotta (4). Calisher et al. (1989) reported that *Flaviviridae* can be divided into eight complexes from which one is *Dengue* with four antigenically different serotypes (5). Kapoor et al. (1996) described the genome of *Dengue* virus as capped RNA capable to produce a poly protein with approximately 3391 amino acids. This poly protein can then be cleaved to three structural proteins (Capsid, envelope, and precursor to membrane) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (6). It is known that *Dengue* virus is transmitted by mosquitos especially by *Aedes aegypti* or *Aedes albopictus* (7) but also can be transmitted by *Aedes niveus*, and *Aedes polynesiensis* (8). Most people show no remarkable symptoms after infection with virus. Still, in some patients, the infection can be accompanied with high fever, headache, body aches (therefore break-bone fever), nausea and rash (9, 1). It seems that the infection rate increases every year in the world. WHO has registered over 7.6 million of *Dengue* cases in 2024, from which 16000 with severe clinical signs and over 3000 deaths (10). Since animals can be as reservoir for infection, it is important to consider the *Dengue* infection also as zoonotic diseases. It was reported that non-human primates, bovids, horses, pigs, birds, and rodents can be infected with this virus (11). Diagnosis of Dengue infection is performed by serological examination, molecular techniques like RT-PCR and multiplex PCR (12). In the present study, we present a RT-PCR and nested-RT-multiplex PCR for simultaneously detection of *Dengue* virus serotypes and discrimination of serotypes from each other.

Materials and Methods

Preparation of Dengue virus templates and samples

The recombinant pEX-A128 plasmids with approximately 120 to 780 nucleotide of 5` region of four *Dengue* virus serotypes were kindly provided by MBST Company (Iran, Tehran) for the present study. The corresponding nucleotide sequences of the mentioned region of four serotypes was derived from accession numbers JQ287663.1, LC707382.1, NCBI Reference Sequence NC_001477.1, OM281599.1, ON632080.1ON631283.1, OP809580.1, OQ714403.1, OR389290.1, OR389304.1, U88536.1 for serotype 1, EU687214.1, KF955345.1, OQ821501.1, OR150743.1, OR616482.1, PP269909.1, PP320855.1, PP764465.1 for serotype 2, MH544650.1, PP711274.1, EU052796.1, DQ109298.1, LC436676.1, KU509280.1, EF629373.1for serotype 3 and AF326573.1, AF326827.1, AF326826.1, AY648301.1, KJ579245.1, KU523872.1, KU523871.1 for serotype 4. These constructs were used as template for amplification of each *Dengue* virus serotype. The *Aedes* mosquito and *Larvae* were kindly provided by Mr. Amin Mozafaripoor from Company Pishgaman Danesh Zhen (Ahwaz, Iran). The identification of *Aedes* mosquito was performed by Department of Parasitology, University of Tehran, based on the white spots against black background of the legs and on the body. The larvae were identified based on the location, size and color of siphon (shorter and darker) in abdomen (13-15).

PCR and Multiplex PCR

The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were transferred into competent *E. coli* DH5α separately and the transfected *E. coli* was grown. Subsequently, the plasmid was extracted with the Plasmid Isolation Kit (MBST, Tehran, Iran). The isolated plasmids were then used as templates for each serotype by PCR and multiplex PCR. For designing of the primers, the corresponding sequences of each serotype (see the accession numbers above) were aligned with each other and a consensus sequence was chosen for each serotype. In the present study, we have checked the primers described by Kumar et al. (2018) (17). The nucleotide sequence of the

consensus sequence of serotypes was aligned together. The designed primers were checked with the corresponding nucleotide alignments of each serotype. Additionally, to the study reported by Kumar et al. (2018) (17), we designed a reverse primer (Dengue all R) having the possibility to amplify all serotypes with the common forward primer (Dengue all F). The primers are listed in the table 1. PCR was first performed using primers Dengue all F and Dengue all R with the extracted recombinant plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3), and D4 (serotype 4)] to obtain a PCR product of 657 bp in length. For this aim, 1µl plasmid (1 ng), 12.5 µl master mix (CinaClone, Iran), 0.5 µl of each Dengue all F and Dengue all R primers (20 µM working solution) in 25 µl total volume were used. The PCR was performed with following conditions: initial denaturation of DNA strands at 95 ^{0}C for 5 min followed by 35 cycles of denaturation at 94 ^{0}C for 45 s, annealing at 63 ^{0}C for 45 s, and extension at 72 ^{0}C for 45 s. The PCR reaction was followed by a final 72 ^{0}C for 10 min. PCR products were then analyzed using 2-2.5% agarose gel electrophoresis using ethidium bromide. After that the PCR products (657 bp in length) were diluted to 1:100 and 1 µl from them was amplified with mix of primers (Dengue all F, Dengue 1R, Dengue 2R, Dengue 3 Rand Dengue 4Rcontaining 0.5 µl (20 µM working solution) of each primers Dengue all F, Dengue 1R, Dengue 2R, Dengue 3R and Dengue 4R. PCR was done as described above except for annealing temperature of 60 ^{0}C

RNA Isolation, cDNA synthesis, RT-PCR, and Multiplex-RT-PCR

For Isolation of RNA, the RNA Extraction Kit from MBST (Tehran, Iran) was used as described by manufacturer's instruction. Briefly, after thoroughly evaporation of pool of ethanol fixed larvae, the sample was lysed in 400 µl lysis buffer (LB-PS) and immediately lysed thoroughly using insulin syringe. After that, 6 µl β-mercaptoethanl was added and mixed by vortexing. The mixture was then incubated at70 ^{0}C. After vortexing of 10 seconds at RT, 30 µL RNA Carrier solution was added and mixed thoroughly. After adding of 480 µl Ethanol (99%, Merck, Germany) and mixing by vortexing, the mixture was transferred into the column and centrifuged for 1 minute at 8000 x g. Subsequently, the column was washed with 500 µl wash buffer (WB-PS I) and twice with each with 500 µl wash buffer (WB-PS II) by centrifugation at 8000 x g for 1 minute. After that, the RNA was eluted from the column with 40 µl preheated DEPC H_2O buffer at 70 ^{0}C. For the synthesis of cDNA, cDNA synthesis kit from CinaClone (Iran) using hexa nucleotides and eight µl RNA in total volume of 20 µl was used.

To check the purity of extracted RNA, PCR was done with RNA, cDNA and genomic DNA from human using primer pair derived from a region of the 18S rRNA gene with possibility to amplify the corresponding region of both *Aedes* mosquito- and human genome (table 1). This primer pair seems to be common in the broad spectrum of eukaryotes. We have used previously this primer pair for quantitative analysis of *Leishmania major* infected mice (16). This primer pair have differences to human corresponding region only in two nucleotides, which have no effect on the amplifying of gene fragment resulting in ca. 250 bp in length. The primer pair was checked for lots of *Aedes spp.* Like *Aedes aegypti* (accession no. MG232525.1), *Aedes geniculatus* (accession no. PV083883.1), *Aedes albopic*tus (accession no. PV067939.1), *Aedes notoscriptus* (accession no. PQ112577.1), *Aedes vittatus* (accession no. MG232588.1), *Aedes sudanensis* (accession no. MG232578.1), *Aedes metallicus* (accession no. MG232568.1), *Aedes luteocephalus* (accession no. MG232566.1), *Aedes japonicus* (accession no. MG232565.1), *Aedes hirsutus* (accession no. MG232561.1) and *Aedes geniculatus* (accession no. MG232558.1). After that, the cDNA was amplified using Dengue all F and Dengue all R primers as described above.

Table 1: Primers were designed from *Dengue* virus genome (1-6) and 18S rRNA gene (7-8).

No	Name von primer	Sequence of the primers 5` ----------3`	PCR product (bp)
1	Dengue all F	TCAATATGCTGAAACGCGAGAGAAACCG	
2	Dengue all R	TCCAAGCCCCTTCAGA**B**GACATCCA	657
3	Dengue 1R	CGTCTCAGTGATCCGGGGG or CGTCTCAGTGATCCGGGG	480
4	Dengue 2R	GCCACAAGGGCCATGAACAG	119
5	Dengue 3R	TGGTAATATCATCATGAGACAGA	290
6	Dengue 4R	TGTTGTCTTAAACAAGAGAGGTC	389
7	SSU-18S rRNA F	AGAGGTGAAATTCTTGGACCG	250 bp
8	SSU-18S rRNA R	TTCCGTCAATTCCTTTAAGTTTCA	

Results

Determination of primer specificity

All primer were controlled through primer blast tool under https://www.ncbi.nlm.nih.gov/tools/primer-blast/ with the following conditions: 2 Min total mismatches, 2 Min 3' end mismatches, 5 Defined 3' end region length, 6 Mismatch threshold to ignore targets, 4000 Max target size, 50000 Max number of Blast target sequences, 30000 Blast E value, 7 Blast word size, 500 Max candidate primer pairs, 67 Min PCR product size, 1000 Max PCR product size, 15 Min Primer size, 20 Opt Primer size, 25 Max Primer size, 57 Min Tm, 60 Opt Tm, 63 Max Tm, 3 Max Tm difference, Auto Repeat filter with Low complexity filter. It is to mention that some of the used primers were still reported in other studies (17, 18, 19). The primer blast revealed that the used primers were specific for *Dengue* virus with one exception, namely, the Dengue all forward primer alone can amplify *Hypaurotis quercus* genome (Interactive Listing of American Butterflies) under accession numbers OZ211844.1, OZ211850.1, OZ211874.1, OZ211869.1 resulting in a PCR product of 1204, 1227, 3645 and 3651 bp respectively. The cross reaction of the mentioned primer with *Hypaurotis*, is negligible as it is not relevant in human/mosquito diagnostics of *Dengue* fever/virus. Apart from this, the primers cannot amplify any PCR product for templates prepared from human and arthropods genomic DNA as well as from *Dengue* virus, from templates prepared from genomic RNA and DNA derived viruses.

PCR and Multiplex-PCR with recombinant Dengue serotype pEX-A128 plasmids

The purified recombinant plasmids were amplified using primers Dengue all F and Dengue all R. Figure 1A showed that all four Dengue serotype plasmid constructs could be amplified and the PCR product was as expected 657 bp in length.

In the second experiment, the purified recombinant Dengue serotype pEX-A128 plasmids were amplified using mix of primers containing Dengue all F, Dengue D1R, Dengue 3R and Dengue 4R primers. Figure 1B showed clearly that the primers can specifically amplify the DNA region of the corresponding serotypes and no cross reactivity was detected. The PCR product of serotypes 1, 2, 3 and 4 was 480 bp, 119 bp, 290 bp and 389 bp respectively and were gut discriminable from each other on the agarose gel. Thus, we confirmed the results of Kumar et al. (2018) (17).

In the third experiment, the PCR products derived from *Dengue* virus serotypes achieved by PCR with Dengue all F and Dengue all R primers (figure 2A), were diluted (1:100) and amplified using primer mix (Dengue all F, Dengue 1R, Dengue 2R, Dengue 3R, and D4R). Figure 2B showed that as expected only one matched PCR product for each serotype could be generated. The PCR products driving from D1-, D2-, D3- and D4-recombinant- pEX-A128 plasmids were also 480 bp, 119bp, 290 bp and 389 bp respectively.

In the fourth experiment we used mix of all primers (Dengue all F, Dengue all R, Dengue 1R, Dengue 2R, Dengue 3R and Dengue 4R) for performance of

PCR. Unfortunately, the amplification of all expected PCR products (657 bp, 480 bp, 119bp, 290 bp and 389 bp) was not always reproducible. The figure 3 showed that in the case of Dengue serotype 1 (figure 3 lane D1) no serotype specific DNA band and in the case of Dengue serotype 3 (figure 3 lane D3), the Dengue all DNA band was not detectable.

RT-PCR and Multiplex RT-PCR

The extracted RNA was first amplified using primer pairs derived from the small subunit 18S rRNA to control the purity of the isolated RNA. Figure 4A, lane 2, showed that the isolated RNA cannot be amplified using the above-mentioned primer pair, indicating that the isolated RNA was pure. In the case of positive reaction, the RNA was treated with the DNase I (RNase free) to obtain pure RNA. The cDNA was than amplified using the same primers derived from 18S rRNA gene and as expected a PCR product with 255 bp in length could be amplified (figure 4 lane 3 and 4). Subsequently, the cDNA was amplified using primer pairs Dengue all F and all R and as positive control D1-serotype-recombinant pEX-A128 plasmid was used. In contrast to D1- serotype-recombinant pEX-A128 plasmid, the cDNAs could not be amplified using the used primers, which indicated that the samples were negative (figure 4B).

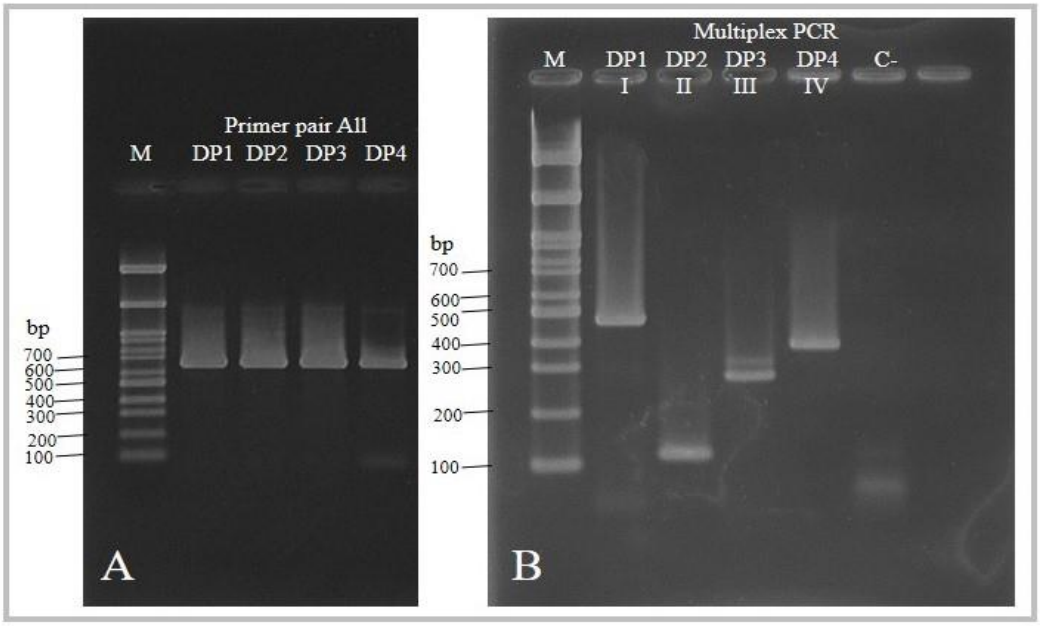

Fig. 1. A: The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were amplified using primer pair Dengue all F and Dengue all R. **B:** The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were amplified using serotype specific primers in multiplex PCR. DP1, DP2, DP3 and DP4 are recombinant Dengue serotype (1, 2, 3 and 4) pEX-A128 plasmid respectively. "C-" is negative control. M is 100 bp DNA marker.

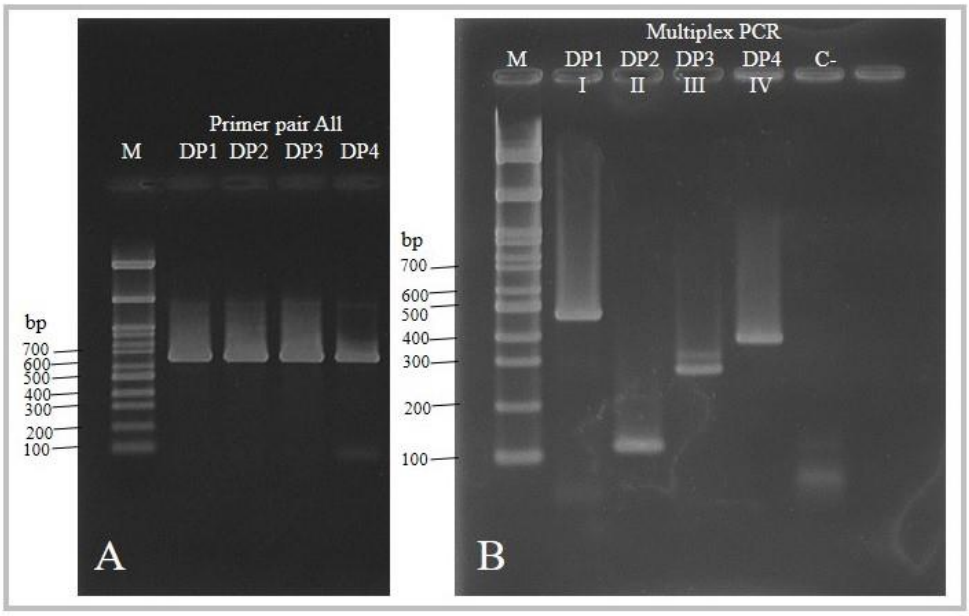

Fig. 2. A: The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were amplified using primer pair Dengue all F and Dengue all R. **B:** The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were diluted (1:100) and 1 µl of them was amplified using serotype specific primers in multiplex PCR. DP1, DP2, DP3 and DP4 are recombinant Dengue serotype (1, 2, 3 and 4) pEX-A128 plasmid respectively. "C-" is negative control. M is a 100 bp DNA marker.

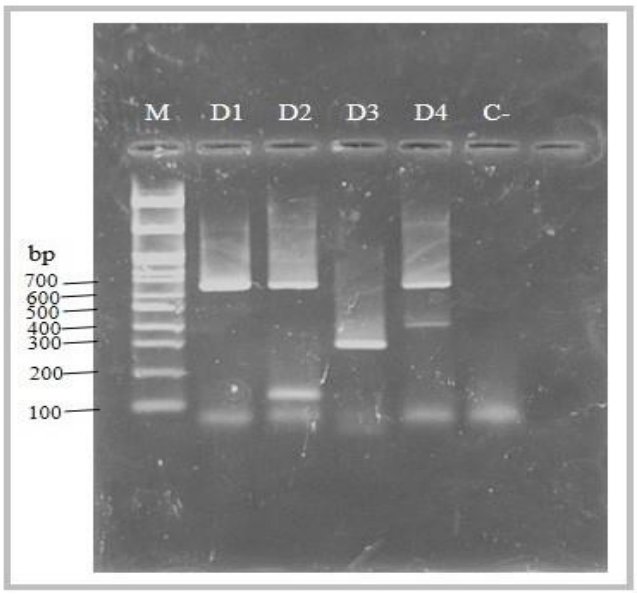

Fig. 3. The recombinant Dengue serotype pEX-A128 plasmids [D1 (serotype 1), D2 (serotype 2), D3 (serotype 3) and D4 (serotype 4)] were amplified using primer pair Dengue all F and Dengue all R. and serotype specific primers in multiplex PCR. D1, D2, D3 and D4 are recombinant Dengue serotype (1, 2, 3 and 4) pEX-A128 plasmid respectively. "C-" is negative controls. M is a 100 bp DNA marker.

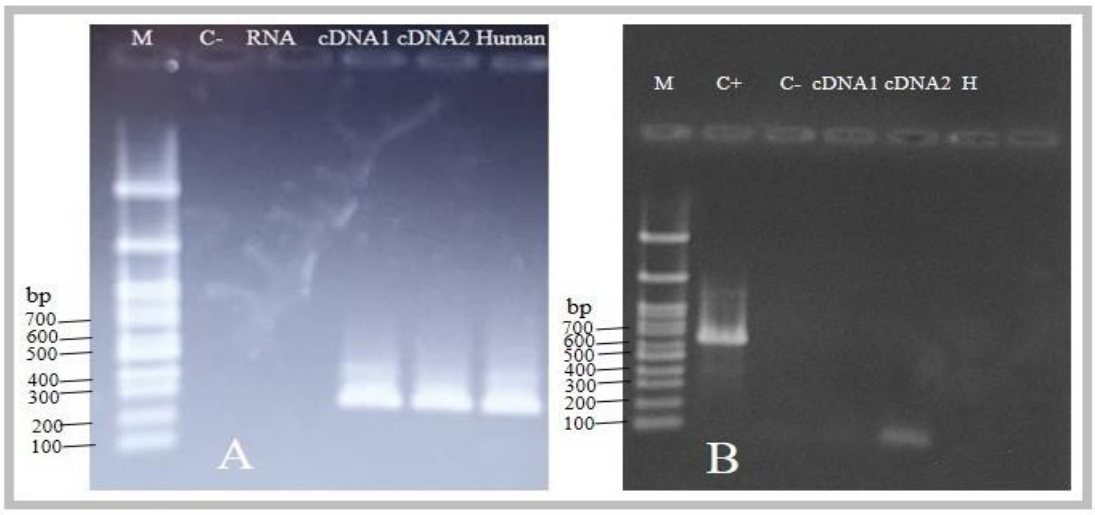

Fig. 4. A: Extracted RNA, cDNA1, cDNA 2 and human genomic DNA were amplified using primer pair derived from 18S rRNA gene (Lane RNA). **B:** The recombinant Dengue serotype D1 pEX-A128 plasmids, cDNA1 and cDNA2, were amplified using Dengue all F and all R.

Discussion

Dengue fever is a disease caused by the dengue virus. *Dengue* virus belongs to the genus *Flavivirus* in the family *Flaviviridae* and has a single-stranded RNA genome with positive polarity (2). The prevalence of *Dengue* virus has increased dramatically worldwide in recent decades. 30 may 2024, WHO reported that over 7.6 million of *Dengue* infection was registered, from which 16000 with severe clinical signs and over 3000 death (10). In Iran, *Dengue* virus became important when Marandi et al. (2013) reported a case report in 2013 of a 58-year-old woman who had traveled to Malaysia 10 days before the onset of symptoms and was infected with *Dengue* virus (20). Heydari et al. (2018) showed that among blood samples from patients suspected of *Dengue* fever in Sistan and Baluchestan province collected between 2013 and 2015, there were positive serological samples against *Dengue* virus (21). Aghaie et al. (2014) reported dengue virus infection among healthy volunteers in Sistan and Baluchestan province using serological methods in 2014 (22). Ebrahimi et al. (2016) also reported in 2016 on the infection of a woman who had lived in Malaysia for 6 years and had returned to the country and had symptoms of dengue fever (23). Also, Baniasadi et al. (2015) reported the infection of a 32-year-old woman in 2015, who had a history of recent travel to India and had symptoms of *Dengue* fever with *Dengue* type 2 virus using serological and RT-PCR and nucleotide sequencing methods (24). They reported that the virus belongs to DENV 2, with a high homology to other strains reported from India. The vector of *Dengue* virus are *Aedes aegypti* and *Aedes albopictus* (7) can also be transmitted by *Aedes niveus*, and *Aedes polynesiensis* (8). It seems that the *Dengue* virus can be considered as an introduced agent in Iran as it was described also by Dabbagh Moghaddam and Bakhtiari (2024) as an uninvited guest (25). They also emphasize that due to the relative suitability of the country's climate for the survival of *Aedes* mosquito and increased travel to areas where the *Dengue* is endemic, it is of very important to implement not only the related educational general public programs but also to monitor the disease. Based on the data from Iranian Ministry of Health and Medical Education, WHO

reported on 22 July 2014 that *Aedes aegypti* and some *Aedes albopictus* mosquitoes *have established themselves* in the provinces of Sistan and Balouchistan, Hormozgan, Bushehr, Khuzastan, and Gilan. Furthermore, this report states also that 12 autochthonous *Dengue* virus infection were documented by 17 July 2024 in Bandar-Lengheh, Hormozgan (26). The early diagnosis of the infection can help to control the disease. US center for Disease control and prevention (CDC) recommended using NS1 ELISA assay as serological test for diagnosis of *Dengue* infection. This organization recommend also the detection of IgM in the serum or plasma and RT-PCR of suspicious patients (27). Many investigators have developed RT-PCR and RT-multiplex PCR test for detection of genomic material of the virus in host specimens. Most of these investigators designed forward primer from the 5` end of the genome of the virus encoding for peptide NMLKRERN which seems to be conserved in *Dengue* virus serotypes. The reverse serotype-specific primers were used differently. Since we do not have available any serotype of *Dengue* virus in our department, we used the *Dengue* serotypes recombinant pEX-A128 plasmids containing 657 bp of 5`region of the genome of the corresponding serotypes. In contrast to Kumar et al. (2018), we first used a reverse primer (Dengue all R) to amplify all four recombinant Dengue serotypes pEX-A128 plasmids simultaneously resulting in PCR product of 657 bp in length (16). Subsequently, the PCR product was diluted 1:100 and 1 µl of which was used in multiplex PCR to detect the serotype of the virus. Additionally, we amplified directly the recombinant Dengue serotype pEX-A128 plasmids in multiplex PCR and could confirm the results achieved by Kumar et al (2018) (16). Belem et al. (2024) used above described common forward primer for serotype 1, 2 and 4, other nucleotide positions for designing reverse primers in their experiments. Furthermore, they used additionally extra forward and reverse primer for amplifying the corresponding gene area for serotype 3 (18). We believe that in developing countries, it is cheaper to use first the RT-PCR for detection of infection and in the case of the positive reaction, the serotype of the virus can be determined by multiplex PCR (RT-nested Multiplex PCR). It is known that an infected person develops protective immunity usually after recovery from an infection with one serotype of virus, but this immunity is not cross-protective immunity. If these people become infected with another serotype, they may be subjected to higher rates of severe symptoms and death (10). Therefore, monitoring the serotypes present in the country is of great importance and the present study can help update the circulating serotypes of *Dengue* virus in Iran.

Conclusion

Prevalence of *Dengue* virus has increased in Iran and public health management through the early diagnosis and determination of the circulating and introduced *Dengue* virus serotype is essential for monitoring the infection and rapid intervention. The presented RT-PCR followed by nested-multiplex PCR can help to realize this intention.

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Conflict of Interests

The authors declare no conflict of interest.

Ethical approval

Not applicable.

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