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Simultaneous detection of Dengue virus, Chikungunya virus, Zika virus, Yellow fever virus and West Nile virus

José A. Boga*, Marta E. Alvarez-Arguelles, Susana Rojo-Alba, Mercedes Rodríguez, María de Oña, Santiago Melón

Servicio de Microbiología, Hospital Universitario Central de Asturias (HUCA) and Instituto de Investigación Sanitaria del Principado de Asturias (ISPA), Oviedo, Spain

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ABSTRACT

Although certain mosquito-borne virus, such as Dengue virus (DENV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Yellow fever virus (YFV) and West Nile virus (WNV), are an important public health concern in those countries where transmitter mosquitoes are endemic, several cases of travelers from those endemic countries have been recently reported in Europe. Thus, early diagnosis of these viruses is essential for patient management and adoption of preventive measures. An assay for the simultaneous detection of DENV, CHIKV, ZIKV, YFV and WNV based on a multiplex real-time (RT)-PCR and its usefulness for diagnosis in infection screenings and surveillance of arbovirus in non-endemic countries are described.

1. Introduction

Dengue virus (DENV), Chikungunya virus (CHIKV), Zika virus (ZIKV), Yellow fever virus (YFV) and West Nile virus (WNV) are mosquito-borne viruses which cause diseases, which range from mild fever to encephalitis and hemorrhagic fevers (Wilder-Smith et al., 2017; Cleton et al., 2012). Although early diagnosis is essential for patient management and adoption of preventive measures, the fact that symptoms often overlap makes difficult to perform a diagnosis based on symptoms alone. Genome detection using techniques based on polymerase chain reaction (PCR) are the best alternative to serological test, which are often nonspecific due to serological cross-reactivity between these viruses (Maeda and Maeda, 2013).

Here, an assay for the simultaneous detection of DENV, CHIKV, ZIKV, YFV and WNV based on a multiplex real-time (RT)-PCR is described.

2. Materials and methods

2.1. Samples and patients

From January to December 2016, 103 samples (52 bloods, 46 urines, 11 pharyngeal swabs and 4 nasopharyngeal swabs) from 59 patients (mean age: 35.1 [19.8] years; range: 5 months – 77 years; 32 males), who came from endemic zones and showed at least one of these symptoms (fever, joint pain and/or conjunctivitis) were collected and transported the same day to our laboratory, where they were stored at 4 °C until their processing. Furthermore, six samples (bloods) belonged to six patients, which had been positive to CHIKV by a "nested" retrotranscriptase-PCRs used routinely in our laboratory during 2014, were used. Purified RNAs from arbovirus which were supplied by the Centro Nacional de Microbiología (CNM, Madrid), were used as positive controls. To test sensitivity and specificity, quantified RNAs from Vircell (Spain) were used. A panel of 18 tubes containing specimens for the detection of arboviruses was supplied by WHO&RCPAQAP.

2.2. Genome detection

All samples were processed to extract acid nucleic by using Ampliprep system (Roche, Germany). Real time (RT)-PCRs were developed to detect DENV, CHIKV, ZIKV, YFV and WNV. Viral genomes were amplified by using TaqMan® Fast Virus 1-Step Master Mix (Life Technologies, CA) and the primers and FAM-labelled MGB probes listed in Table 1. Amplifications and data analysis were performed in a 7500 Real Time PCR System (Applied Biosystems, CA) using the following conditions: retrotranscription at 50 °C for 20 min; denaturation at 95 °C for 5 min; 40 cycles at 95 °C for 10 s, 55 °C for 15 s, and 60 °C for 20 s. Positive controls supplied by CNM were included in all assays.

All human samples were analyzed by a multiplex RT-PCR, where the assay contains all primers and FAM-labeled MGB probes, as well as with five singleplex RT-PCRs, where each assay contains only two primers and one probe, to identify the virus.

* Corresponding author.
E-mail address: joseantonio.boga@sespa.es (J.A. Boga).

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Table 1
Primers and probes used to detect DENV, CHIKV, ZIKV, YFV and WNV.

Virus	Name	Sequence (5'- 3')	Position[a]	Strain[b]
	DEN.ZIK-TR-S	AAGGACTAGAGGTTAGAGGAGACCC	10589-10613	
Dengue	DEN-TR-A	GAGACAGCAGGATCTCTGGTCT	10650-10672	NC_001477
	DEN-FAM	AACAGCATATTGACGCTGG	10628-10646	
	DEN.ZIK-TR-S	AAGGACTAGAGGTTAGAGGAGACCC	10657-10681	
Zika	ZIK-TR-A3	GGCCAGCGTGGTGGAA	10737-10753	NC_035889
	ZIK-FAM	AAACAGCATATTGACG	10695-10710	
	CHK-TR-S	CGGCGACCATTTGTGATCA	1080-1098	
Chikungunya	CHK-TR-A	TTCGTATTCCGTTGCGTTCTG	1186-1206	NC_004162
	CHK-FAM	CCGGCATCCTTGCTA	1104-1118	
	WN.FA-TR-S	GGCTGGGGCAAYGGCT	1271-1286	
Yellow fever	FA-TR-A	CCTCAAACAAACTCATGGATTTG	1339-1361	NC_002031
	WN-FA-FAM	CATGCGCCAAATT	1317-1329	
	WN.FA-TR-S	GGCTGGGGCAAYGGCT	1264-1279	
West Nile	WN-TR-A	CAAGATGGTTCTTCCTATTGCCT	1237-1358	NC_009942
	WN-FA-FAM	CATGCGCCAAATT	1211-1322	

[a] Positions correspond to nucleotide sequences of reference strains.
[b] Genbank accession number of reference strains.

3. Results

Sensitivity of the all RT-PCRs was established by using commercially available purified RNAs (Vircell, Spain) for each virus resulting that at least 50 copies of each viral genome were detected in multiplex RT-PCR, as well as in the five singleplex RT-PCR.

A panel of arboviruses supplied by WHO&RCPAQAP was also tested. Negative and positive controls were included in all PCR reactions performed. The results are shown in Table 2. In 17 (94.4%) of the samples, a correct result was obtained, including two samples with a mixed infection. All the four genotypes of DENV, as well as the two lineages of ZIKV were detected. No virus was detected in two negative samples and in one Japanese Encephalitis Virus (JEV)-containing sample.

To test assay exclusitvity with other flavivirus, JEV sample present in the arbovirus panel and Tick-Borne Encephalitis Virus (TBEV) purified RNA (Vircell, Spain) was tested. No cross-reactions with these flaviviruses were detected.

Finally, 109 clinical samples were used to test the assay. Of them, 103 samples were collected during 2016 and the remaining 6 samples belonged to six patients, which had been positive to CHIKV by a "nested" retrotranscriptase-PCRs used routinely in our laboratory during 2014. All of them were analyzed with the multiplex RT-PCR, as well as with the five singleplex RT-PCRs to identify the virus (Table 3). All CHIKV-positive samples were confirmed. No CHIKV, YFV and WNV were detected in the 103 samples collected during 2016. Nevertheless, 10 (9.71%) samples (6 urines, 3 pharyngeal swabs and 1 blood) belonging to 6 (10.2%) patients and 3 (2.91%) samples (2 bloods and 1 pharyngeal swab) belonging to 2 (3.39%) patients were positive for ZIKV and DENV, respectively. Demographic characteristics and clinical symptoms of infected patients are shown in Table 3.

Table 2
Virus detected by multiplex and singleplex RT-PCRs in the arbovirus panel supplied by WHO&RCPAQAP.

Specimen	WHO&RCPAQAP	Multiplex and singleplex RT-PCRs		
	Analyte	Analyte	CTm[a]	CTs[b]
ARBO-01:2016	YFV	YFV	21	25
	CHIKV	CHIKV		22
ARBO-02:2016	Negative	Negative	–	–
ARBO-03:2016	CHIKV	CHIKV	23	22
ARBO-04:2016	ZIKV Asian lineage	ZIKV	30	27
ARBO-05:2016	ZIKV Asian lineage	ZIKV	30	24
ARBO-06:2016	DENV type 3	DENV	24	27
	ZIKV Asian lineage	ZIKV		28
ARBO-07:2016	DENV type 1	DENV	22	21
ARBO-08:2016	ZIKV African lineage	ZIKV	26	22
ARBO-09:2016	DENV type 4	DENV	25	23
ARBO-10:2016	WNV	DENV	36	36
ARBO-11:2016	JEV	Negative	–	–
ARBO-12:2016	ZIKV Asian lineage	ZIKV	34	32
ARBO-13:2016	DENV type 2	DENV	19	18
YFV-01:2016	YFV 17D	YFV	26	23
YFV-02:2016	ZIKV African lineage	ZIKV	24	22
YFV-03:2016	Negative	Negative	–	–
YFV-04:2016	YFV 17D	YFV	25	24
YFV-05:2016	YFV 17D	YFV	25	24
		DENV		32

[a] CTm: Ct was obtained by the multiplex RT-PCR.
[b] CTs obtained by singleplex RT-PCR; JEV, Japanese Encephalitis Virus.

4. Discussion

Arbovirus including Flavivirus, such as DENV, ZIKV, YFV and WNV, and Alphavirus, such as CHIKV, are an important public health concern in those countries where transmitter mosquitoes are endemic. In Europe, cases of travelers from those endemic countries have been reported (Duijster et al., 2016; Ramos Geldres et al., 2015; van den Bossche et al., 2015). Considering that a fast, efficient, and sensitive method for the diagnosis of these viruses is essential for the management of infected patients, several methods has been developed. Although virus isolation is used for the diagnosis of some of these viruses, this method has low sensitivity and requires several days for completion. The serological tests can cross-react between flaviviruses or have false negative results (Kuno, 2003). The methods based on PCR techniques have many advantages compared with virus isolation and serological assays and are becoming the gold standard for the diagnosis of arbovirus (Calvo et al., 2016; Pabbaraju et al., 2016).

In this study, we report the evaluation of a RT-PCR for the detection of five arboviruses (DENV, CHIKV, ZIKV, YFV and WNV) simultaneously. In our system, samples are first analyzed by a multiplex RT-PCR, which can identify all viruses together. Positive samples are then analyzed by the five singleplex RT-PCRs to identify the virus or viruses present in the sample. Although several detection methods based on genome detection of some of these viruses have been reported, our method is the only to detect simultaneously five of the most prevalent arbovirus. Furthermore, our diagnostic algorithm, which consists in a first screening to detect all viruses followed by a second specific detection method used only in the first screening-positive samples, is a fast and relatively cheap method to diagnose these infections in non-endemic zones, where major of analyzed samples are expected to be negative. Another advantage of our method is that new primers and probes directed to other arbovirus can be added to the multiplex PCR increasing the number of viruses detected in the first screening.

Table 3
Demographic characteristics and clinical symptoms of arbovirus-infected patients.

Patient	Age	Sex	Symptoms	Date	Sample	Genome detection		
						Virus	CTm[a]	CTs[b]
1	48	M	Fever, joint pain after travel to Puerto Rico	21.07.2014	Blood	CHKV	29	27
				06.08.2014	Blood	Neg		
2	26	F	Fever, after travel to Puerto Rico	25.07.2014	Blood	CHKV	33	31
3	55	F	Fever, after travel to Puerto Rico	29.07.2014	Blood	CHKV	28	25
4	71	M	Fever joint pain after travel to Cuba	22.08.2014	Blood	CHKV	27	25
5	51	M	Fever after travel to Laos	10.09.2014	Blood	CHKV	32	30
6	37	M	Fever, joint pain after travel to India	15.09.2014	Blood	CHKV	30	26
				10.10.2014	Blood	Neg		
7	37	F	Fever after travel to Venezuela	22.01.2016	Urine	ZIKV	38	33
					Blood	Neg		
8	10	F	Fever after travel to Venezuela	22.01.2016	Urine	ZIKV	36	34
					Blood	Neg		
9	32	M	Fever after travel to Cuba	27.01.2016	Blood	DENV	30	28
10	12	F	Fever	09.02.2016	Pharyngeal	DENV	33	31
					Blood	DENV	18	17
11	7	F	Fever after travel to El Salvador	30.07.2016	Urine	ZIKV	31	30
					Pharyngeal	ZIKV	37	35
					Blood	Neg		
12	43	F	Fever, rash after travel to Nicaragua	10.08.2016	Urine	ZIKV	27	24
					Pharyngeal	ZIKV	32	30
					Blood	Neg		
13	41	M	Fever, rash after travel to Nicaragua	10.08.2016	Urine	ZIKV	36	34
					Pharyngeal	ZIKV	33	31
					Blood	Neg		
14	37	F	Fever, rash after travel to Mexico	23.08.2016	Urine	ZIKV	32	28
					Blood	ZIKV	33	26

[a] CTm: Ct was obtained by the multiplex RT-PCR.
[b] CTs: Ct was obtained by singleplex RT-PCR;Neg, Negative.

In summary, the RT-PCR method proposed in this report may be useful for diagnosis in infection screenings and surveillance of arbovirus in non-endemic countries.

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