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Easy and inexpensive molecular detection of dengue, chikungunya and zika viruses in febrile patients

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ABSTRACT

Dengue (DENV), chikungunya (CHIKV) and zika (ZIKV) are arthropod-borne viruses (arboviruses) sharing a common vector, the mosquito *Aedes aegypti*. At initial stages, patients infected with these viruses have similar clinical manifestations, however, the outcomes and clinical management of these diseases are different, for this reason early and accurate identification of the causative virus is necessary. This paper reports the development of a rapid and specific nested-PCR for detection of DENV, CHIKV and ZIKV infection in the same sample. A set of six outer primers targeting the C-preM, E1, and E gene respectively was used in a multiplex one-step RT-PCR assay, followed by the second round of amplification with specific inner primers for each virus. The specificity of the present assay was validated with positive and negative serum samples for viruses and supernatants of infected cells. The assay was tested using clinical samples from febrile patients. In these samples, we detected mono and dual infections and a case of triple co-infection DENV-CHIKV-ZIKV. This assay might be a useful and an inexpensive tool for detection of these infections in regions where these arboviruses co-circulate.

1. Introduction

Arthropod-borne viruses (arboviruses) are the causal agents of clinical and subclinical infections in humans, known as non-specific fever illness (Vasconcelos and Calisher, 2016), which represent one of the most important public health concerns in tropical and subtropical areas of the globe. Dengue virus (DENV), along with the recently introduced chikungunya virus (CHIKV) and Zika virus (ZIKV), are currently the most prevalent arboviruses in the Americas (Rodriguez-Morales, 2015).

There are four DENV serotypes (DENV 1–4); therefore, an individual can be infected up to four times with DENV. Dengue occurs in nearly 100 countries; approximately 50 million individuals are infected annually, and more than 500,000 patients develop severe dengue infections (Bhatt et al., 2013) that frequently require hospital admission to manage the plasma leakage, fluid accumulation, respiratory difficulties, hemorrhages and organ damage that are characteristics of this condition. In the early 1980s, dengue spread throughout most countries in the Americas and subsequently has increased in circulation to reach 2.5 million cases in 2015, including 10,276 severe cases and 1174 fatalities (PAHO, 2016a). Brazil, Mexico and Colombia account for nearly 70% of the cases in the Americas.

Although CHIKV outbreaks have been reported since the 1950s in Africa and Asia, it was not until 2013 that the virus started to circulate in the Americas. From 2013 to December 2015, 1.8 million cases and 265 fatalities have been reported in the Americas (PAHO 2014, 2016b). The Dominican Republic (540,000 cases in 2014) and Colombia (356,000 cases in 2015) were the most affected countries, although Martinica, the Guadeloupe islands and Colombia were the territories that reported the highest number of fatal cases of CHIKV (83, 70 and 57, respectively) (PAHO, 2014, 2016b).

Prior to 2007, ZIKV infection and disease had only been reported sporadically in Africa and Asia, even though the virus had been first isolated in African monkeys in 1947 (Dick et al., 1952). It was not until 2007 that the first epidemic outbreak in Yap Island – Federated States of Micronesia- was described (Duffy et al., 2009), and more recent epidemic outbreaks were reported in French Polynesia and other Pacific islands in 2013 (Cao-Lormeau et al., 2014; Musso et al., 2014). In 2015, it started to circulate in the Americas and spread to 20 countries and territories (Hennessey et al., 2016). It

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has been estimated that between 440,000 and 1.3 million cases occurred in 2015 in Brazil, which has to date been the most affected country (ECDC, 2016). Since the confirmation of ZIKV circulation in Colombia (November 2015) until epidemiological week 17 of 2016, there have been 71,000 reported clinical cases (Sivigila, 2016).

These three viruses can be transmitted to humans by the bite of *Aedes* genus mosquitoes (*Ae. aegypti* and *Ae. albopictus*); therefore, it may be expected that co-circulation and possibly simultaneous infection with two or three arboviruses may occur (Musso et al., 2015). Classical symptoms associated with these three viruses include fever, rash, headache, myalgias and arthralgias, which makes clinical diagnosis during the acute phase of infection difficult. However, each illness has a different course. DENV infection has a wide range of clinical presentations, ranging from mild to fatal disease (WHO, 2009). CHIKV-associated fevers are rarely fatal, but they can trigger a chronic rheumatic syndrome or neurological injury with permanent disability in newborns (Economopoulou et al., 2009; Gérardin et al., 2014). For ZIKV infection, during the French Polynesia outbreak, illnesses were associated with Guillain–Barré Syndrome, meningitis and meningoencephalitis (Cao-Lormeau et al., 2016; Ioos et al., 2014), while during the Brazil outbreak, infection of pregnant women appeared be related to birth defects, such as microcephaly (Kleber de Oliveira et al., 2016; Mlakar et al., 2016) and other neurological manifestations, and congenital abnormalities (Carod-Artal, 2016; Dyer, 2015; PAHO/WHO, 2015).

Differential diagnosis of these arboviruses is very wide and distinguishing the cause during the acute phase based on clinical signs or symptoms is not possible. In Latin American countries, laboratory tests to confirm infection are restricted to DENV (Castellanos and Coronel-Ruiz, 2014). Although laboratory tests are available to detect CHIKV genomic RNA or antigens (Panning et al., 2009; Prat et al., 2014; Yap et al., 2010), these tests are not routinely used in diagnoses. The confirmation of ZIKV cases is even more difficult because there are no commercial or validated tests available, so confirmation is limited to reference laboratories where a small number of samples are sent.

Molecular tests have been frequently and successfully used to diagnose infectious diseases, and they show greater sensitivity and specificity than serological tests. Moreover, because of the absence of a universal test for ZIKV detection, the present study aimed to standardize a nested PCR to simultaneously detect DENV, CHIKV and ZIKV in serum samples from febrile patients with a presumptive diagnosis of one of these viruses. Such a conventional PCR assay could be used as a routine test in most diagnostic laboratories.

2. Materials and methods

2.1. RNA isolation

Using 140 μL of serum or plasma, RNA was isolated using a QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Before the detection of viruses, all the samples were processed to evaluate RNA quality, amplifying by RT-PCR a non coding RNA (extraction control).

2.2. ZIKV detection by nested PCR

ZIKV sequences that had been previously reported from cases diagnosed in the Americas were used to analyze and modify specific primers reported before (Faye et al., 2008, 2013; Lanciotti et al., 2008); oligos chosen are listed in Table 1. The usefulness of the F944–R1269 primers to detect ZIKV was tested in serum from a previously confirmed case. First round amplification was performed with SuperScript III One-Step RT-PCR kit (Invitrogen) using 5 μL of RNA and 0.2 μM outer primers. RNA isolated from DENV-infected C6/36 mosquito cells and patient serum samples obtained in 2014, before ZIKV started to circulate were used to evaluate specificity. Second-round amplification was carried out using 2 μL of the first amplification product as a template and 0.2 μM inner ZIKV primers F944–R1241. Amplification products were separated and analyzed on a 2% agarose gel that was stained with ethidium bromide. Amplicon identity was confirmed by sequencing.

2.3. DENV, CHIKV and ZIKV detection

2.3.1. Reverse transcription and first-round multiplex amplification

RT-PCR was carried out in a 20 μL final volume using the SuperScript III One-Step RT-PCR kit (Invitrogen), 5 μL of template RNA (50–80 ng/μL) and 0.2 μM of each of the following primer pairs for each virus (i.e., a total of six primers): CHIKVF10240–R10540; DENV mD1–D2; and ZIKV F944–R1269 (Table 1). The amplification profile was as follows: 15 min at 50 °C, 5 min at 95 °C and 30 three-step cycles (95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s). A positive control for DENV was RNA isolated from C6/36 infected cells with each one of the four serotypes. The CHIKV positive control was RNA obtained from Vero cell supernatants that had been inoculated with the CHKB64 isolate; positive test results were confirmed by sequencing. During the standardization process, RNA isolated from previously confirmed samples was classified as DENV or CHIKV and then used as positive controls.

2.3.2. Second-round amplification

Specific detection of each virus was carried out in separate reaction tubes using 2 μL of first-round product as a template, 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 0.2 μM of specific primer pairs for each virus (or five primers used for DENV serotyping). Thermocycling conditions were as follows: 25 three-step cycles of 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Reations were performed on a T100 thermal cycler system (BioRad, United States). Amplification products were analyzed in ethidium bromide-stained 2% agarose gels.

3. Results

3.1. ZIKV detection by nested PCR

Specific primers designed to anneal to the 5′-region of the ZIKV E gene robustly recognized the new Colombian isolates without amplifying the four DENV strains (Fig. 1). The ZIKV F944–R1241 primers used in the nested PCR yielded an amplicon of the predicted size (297 bp) from a previously confirmed sample. The 297 bp amplicon showed 100% identity with the sequence KU922923.1 isolated from a Mexican patient.

3.2. Specificity of arbovirus nested PCR

After standardizing the ZIKV detection protocol, we established a multiplex RT-PCR to detect CHIKV, DENV and ZIKV in febrile patient samples. After the first round of amplification, there was only detectable amplicon in the positive controls (DENV or CHIKV culture supernatants) but not in the RNA from serum samples (data not shown). However, after the second round of amplification (nested amplification) using specific primers for each virus, amplicons of the expected size for CHIKV (204 bp) and ZIKV (297 bp) were detected, while for DENV, the size corresponded to the serotype. Bands only appeared in the respective controls and in serum samples that had been previously confirmed for each virus in a uniplex reaction (Fig. 2). We observed no cross reactivity between the different viral RNAs and no interference between the six primers used