Improvement of a RT-PCR assay for Yellow Fever virus genome detection

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ABSTRACT
The aim of the present study was to describe an improved protocol of reverse transcription polymerase chain reaction (RT-PCR) for Yellow Fever virus genome detection. A strain of ribonucleic acid of Yellow Fever virus was submitted to the improved protocol of RT-PCR and the amplicons were visualized under ultraviolet transilluminator, purified and sequenced. The nucleotide sequence obtained was compared with sequences available in GenBank using the tblastx tool. The amplicons produced by the strain of ribonucleic acid of Yellow Fever virus exhibited fragments of 400 and 800 base pairs and the consensus sequence exhibited a similarity of 100% with Yellow Fever virus sequences recorded in GenBank. The improved protocol described in this study allowed Yellow Fever virus genome detection and enabled the elimination of the nested-PCR step, which has been frequently associated with contamination. In addition, it reduced the time of reaction, the cost of reagents and the possibility of sample contamination. New methods of investigating these infections must be elaborated and a continuous vigilance of these viruses in their different vectors and hosts is required to avoid negative impacts on human health, tourism and trade.

Keywords: Yellow Fever virus. Flavivirus. Reverse Transcriptase Polymerase Chain Reaction.

The arboviruses that are most relevant to public health belong to the Flaviviridae family, of which the Flavivirus genus is the most representative, with 53 species recognized by the International Committee on the Taxonomy of Viruses (ICTV, 2014). The genome of these viruses consists of single stranded RNA with positive polarity (Rice et al., 1985).

Flaviviruses can cause encephalitis, hemorrhage, severe fever and hepatic diseases in vertebrates, including humans, and exhibits high morbidity and mortality rates (Monath & Heinz, 1996). Thus, the Flavivirus genus includes some of the most important pathogenic arboviruses worldwide (Heinz & Stiasny, 2012).

In Brazil, more than 10 species of Flavivirus have been isolated from mosquitoes, others animals and humans, including: Yellow Fever virus, Dengue virus, West Nile virus, Saint Louis encephalitis virus, Zika virus, Bussuquara, Cacipacore, Iguape, Ilheus and Rocio virus. According to the Brazilian Ministry of Health, 405 human cases of Yellow Fever were recorded over a period of fourteen years (1999-2013) with 182 deaths, presenting a high lethality rate (44.9%) (Brasil, 2015).

Most epidemiological data concerning cases of Flavivirus infection are underestimated due to the fact that vigilance is notoriously insensitive in endemic areas (Gubler, 2004).

The clinical differential diagnosis of Flavivirus infection is generally complex, particularly in the acute phase of the disease, due to the presence of non-specific symptoms (Moraes-Bronzoni et al., 2005). Most diagnoses are made by serological tests, however, these tests still rely on virus isolation. Despite being widely used, in many cases these techniques are considered incompatible with laboratory routine and unsuitable for processing samples on a large scale, such as during periods of outbreaks (Meiyu et al., 1997; Moreli et al., 2002). In this context, the implementation of virological methods may play an important role in Flavivirus infections diagnosis (Araújo et al., 2012) and in Flavivirus surveillance.
Among molecular biology techniques, reverse transcription polymerase chain reaction (RT-PCR), is considered by many authors as a faster, safer, more sensitive and more specific technique than the usual tests for viral identification (Eldadah et al., 1991; Tanaka 1993; Seah et al., 1995; Scaramozzino et al., 2001; Ayers et al., 2006).

A single polyvalent PCR reaction can be used to detect different strains of Flavivirus in different sources such as vectors and vertebrate hosts and enable the monitoring of viral activity (Ayers et al., 2006). A rapid identification of the pathogenic agent of epidemics contributes to the adoption of appropriate control strategies (Pierre et al., 1994).

The aim of the present study was to describe an improved protocol of RT-PCR assay for Yellow Fever virus genome detection based on electrophoretic migration patterns of amplified fragments and gene sequencing.

A 17D vaccine strain of ribonucleic acid (RNA) of Yellow Fever virus (YFV) provided by Evandro Chagas Institute (Ananindeua-PA-Brazil) and primers Flav100F (5´AAYTCIACICAIGARATGTAY3´) and Flav200R (5´CCIARCCACATRWACCA3´) (Maher-Sturgess et al., 2008) were used in the present study.

In order to obtain cDNA; 2,000 ng of viral RNA (YFV) and 1μL of the primer Flav 200R (50pmol) were added to a sterile microvial and incubated at 70°C for 5 min in a thermocycler (Amplitherm – Thermal Cycler, USA). Subsequently, the sample was placed in an ice bath and 5μL of buffer 5x, 0.5 μL of dNTPs (200mM) and 20U of the AMV reverse transcriptase enzyme (Promega, USA) were added. The final volume of the mix was adjusted to 25 μL with ultrapure water, and the sample was then placed in the thermal cycler again for 90 min at 42°C and 15 min at 70°C. The negative control contained ultrapure water instead of RNA.

The amplification reaction was performed using 3 μL of cDNA, 2.5 μL of 10x buffer, 1 μL of each primer (20pmol), 1.5 μL of MgCl2 (25mM), 0.5 μL of dNTPs (200mM) and 3U of AmpliTaq Gold DNA polymerase (Applied Biosystems, USA). The final volume of the reaction was adjusted to 25 μL with ultrapure water. The sample then returned to the thermocycler for 35 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 2 min. The negative control contained ultrapure water instead of cDNA. The protocol established in this study was adapted from Bona et al. (2012).

The PCR product was subjected to electrophoresis in 2% agarose gel and then visualized on UV transilluminator. The PCR product of the 17D vaccine strain of ribonucleic acid of Yellow Fever virus exhibited fragments of 400 and 800 base pairs (Figure 1) and the consensus sequence exhibited a similarity of 100% with Yellow Fever virus sequences available in GenBank (Figure 2).

Considering the incidence and the emergent importance of diseases caused by Flavivirus, the detection and identification of these viruses and a correct diagnosis of these infections has acquired great importance (Ayers et al., 2006).

Flaviviruses infections are usually identified through serological tests such as complement fixation, neutralization, hemagglutination inhibition, and the immunofluorescence antibody test (Calisher et al., 1989). However, due to similar features exhibited by different Flaviviruses, cross-reactions may occur. This may be a significant problem in the specific diagnosis of these infections, especially when diagnosed through immunoenzymatic or immunofluorescent assays (Heinz & Stiasny, 2012). Especially in infections with different Dengue serotypes, a specific serodiagnosis is difficult and may require different types of immunoassays.
Thus, further studies to detect and identify these viruses in their vertebrate hosts and vectors are needed to enable a prediction of epidemics in humans, avoiding negative impacts on health, tourism and trade.

ACKNOWLEDGEMENTS

We thank Evandro Chagas Institute, Laboratory of Veterinary and Medical Entomology and Pharmaceutical Sciences Graduate Program – Universidade Federal do Paraná (UFPR).

Coordination of Training of Higher Education Graduate Foundation [Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES)] for providing the financial support and Fundação Araucária, Pharmaceutical Sciences graduate program – Universidade Federal do Paraná.

RESUMO

Aperfeiçoamento de um ensaio de RT-PCR para detecção do genoma do vírus da Febre Amarela

O objetivo do presente estudo foi descrever o aperfeiçoamento de um protocolo de reação de transcrição reversa seguida da reação em cadeia da polimerase (RT-PCR) – que foi utilizado anteriormente para detectar e identificar os diferentes sorotipos do vírus da dengue – para a detecção do genoma do vírus da Febre Amarela. Uma cepa do ácido ribonucleico do vírus da Febre Amarela foi submetida ao protocolo de RT-PCR adaptado e os fragmentos amplificados foram visualizados por meio de transiluminador ultravioleta, purificados e sequenciados. As sequências de nücleotídeos obtidas foram comparadas com sequências depositadas no Genbank utilizando a ferramenta tblastx. Os fragmentos amplificados pela cepa do vírus da Febre Amarela exibiram fragmentos de 400 e 800 pares de bases e as sequências consenso exibiram uma similaridade de 100% com sequências depositadas no Genbank. O protocolo adaptado descrito neste estudo permitiu a detecção do genoma do vírus da Febre Amarela e possibilitou a eliminação da etapa nested-PCR que está frequentemente associada com contaminações. Além disso, reduziu-se o tempo de reação, o custo com reagentes e a possibilidade de contaminação da amostra. Novos métodos de investigação destas infecções devem ser elaborados além da vigilância contínua destes vírus a fim de evitar impactos negativos sobre a saúde, o turismo e o comércio.

Improving RT-PCR to detect YF virus

REFERENCES


Received for publication on 26th January 2016
Accepted for publication on 04th August 2017