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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.

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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 70ºC 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 42ºC and 15 min at 70ºC. 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 purification reaction was performed using the QIAquick PCR Purification Kit (Qiagen, USA), following the manufacturer's instructions. The purified PCR product was sequenced in the ABI3730 DNA Analyzer automatic sequencer (Applied Biosystems, USA). The consensus sequence was obtained using the Staden package version 1.5. The nucleotide sequence obtained in the present study was compared with sequences available in GenBank using the tblastx tool.

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).

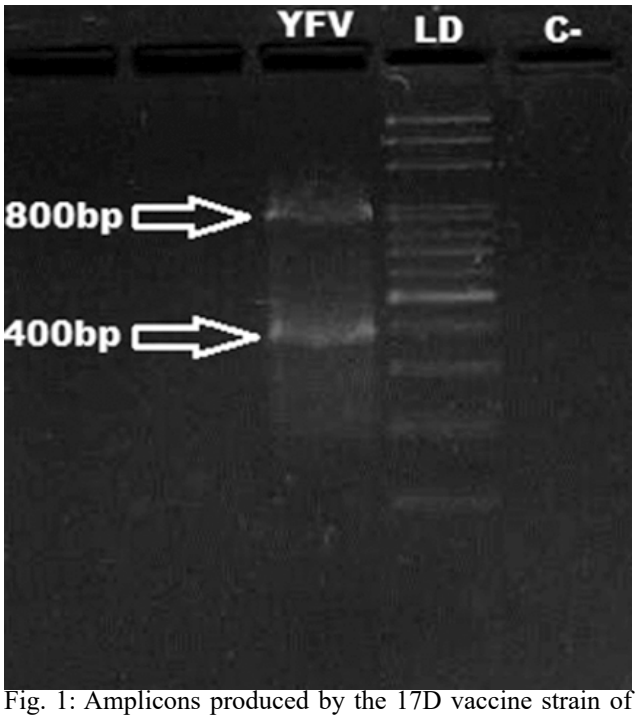

Fig. 1: Amplicons produced by the 17D vaccine strain of ribonucleic acid of Yellow Fever virus, 2% agarose gel visualized on UV transilluminator. Note: LD (Ladder): 100 base pairs, C-: Negative control.

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ATTGGAACAC GCAGTGTGGA AACAGACAAG GGACCACTTG AAAGGGCAGC
CATTGAAGAG AGGGTTGAAA GAATAAAATC TGAATATACT GCCACTTGGT
TCTATGACAA TGACAATCCC TACAGGACCT GGCATTACTG CGGCTCTTAT
GTCACAAGAA CTTCAGGGAG CGCAGCAAGC ATGATTAACG GGGTGATCAA
AATTCTGACA TACCCCTGGG ATAGGATAGA GGAAGTCACG GGAAAAAGTT
TGACAGACAC AACTCCTTTT GGACAACAAA GAGTGTTCAA GGAAAAAGTT
GACACCAGAG CAAAGGATCC ACCATCAGGG ACGAGGAAAA   TCATGA
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Fig. 2: Consensus sequence of the 17D vaccine strain of ribonucleic acid of Yellow Fever virus.

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

(Heinz & Stiasny, 2012). There is also the possibility of negative results during the first days of infection (Moraes-Bronzoni et al., 2005).

Serological methods require a long time to execute and are considered laborious techniques that require specialized professional knowledge in order to isolate and identify the virus (Meiyu et al., 1997; Maher-Sturgess et al., 2008). Although viral isolation through cellular cultivation has been widely utilized, it is a laborious method, which is biased for viruses that exhibit good *in vitro* growth (Pabbaraju et al., 2009).

The development of molecular methods, including RT-PCR, to detect and identify these pathogens has been widely studied (Ayers et al., 2006). This technique (RT-PCR) offers several advantages in detection, identification and diagnosing these viral infections. It is specific, sensitive, safe and can be used to detect different viruses (Eldadah et al., 1991). In addition, it is a faster and easier technique, providing results in up to eight hours. Conversely, serological methods depend on viral isolation (4 to 5 days) and require at least 10 to 14 days to detect antibodies in serum (Seah et al., 1995).

The methodology described in this study is a result of an adaptation of a RT-PCR protocol that was originally used to detect and identify different serotypes of Dengue virus. Therefore, the original primers (species-specific and serotype-specific) were changed by consensus primers. This adaptation allowed the detection of Yellow Fever virus genome and allowed the elimination of nested-PCR step, which has been frequently associated with contaminations (Nunes et al., 2012). In addition, it reduced the time of reaction, the cost of reagents and the possibility of sample contamination.

The RT-PCR assay can utilize specific primers or consensus primers. Consensus primers are developed to amplify genetically related viruses. In these assays, primers are created from highly conserved regions of the viruses genome. This approach offers a method of screening a large quantity of samples.

Molecular methods are efficient and allow the immediate detection and identification of the pathogen in both biological samples and vectors. This is significant because it can provide the diagnosis of the infection during the acute phase of the disease and enable an early detection of the etiological agent (the cause of outbreaks is often unknown) (Eldadah et al., 1991; Kuno, 1998). The arbovirus surveillance in different vertebrate hosts and vectors provides a positive impact on human disease prevention, considering that the results may stimulate appropriate public health actions for prevention (e.g., vaccination and control of vectors) (Lanciotti, 2003).

Due to the ability of Flaviviruses to expand their geographic area and due to the severity of *Flavivirus* infections, new methods of diagnosing these infections must be elaborated and a continuous vigilance of these viruses is required in different sources as vectors and hosts.

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.

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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 nucleotídeos 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.

Palavras-chave: Vírus da Febre Amarela. Flavivírus. Reação de Transcriptase Reversa seguida da Reação em Cadeia da Polimerase.

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