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Comparative theoretical-experimental study applied to the analysis of the gene expression of DNA forms in arthropods and mammalian cells infected by Dengue Vírus

Estudo teórico-experimental comparativo aplicado à análise da expressão gênica de formas de DNA em células de artrópodes e de mamíferos infectadas experimentalmente pelo Vírus Dengue

Estudio comparativo teórico-experimental aplicado al análisis de la expresion gênica del formas de DNA en artrópodos y células de mamíferos infectadas por el Vírus del Dengue

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Abstract

Objective: A study with a theoretical-experimental approach applied to the gene expression of DENV DNA forms in mammalian cells, compared to the presence of fragments originating from viral RNA described in the literature in arthropod cells, experimentally infected by DENV. Methodology: This is a bilateral study, of a theoretical-experimental nature, with a primary intervention basis, carried out in vitro, under a comparative technical approach. Laboratory techniques for gene expression by conventional PCR, verification procedures for analysis and literature data records were obtained and processed at the Viral Biogenesis Laboratory, linked to the Arbovirology and Hemorrhagic Fevers Sector of the Evandro Chagas Institute. Results: Experimental analysis indicated the presence of DENV-4 DNA forms in cells persistently infected by inoculation at 72 hpi. As a comparative function, C6/36 cells presented viral DNA sequences incorporated in regions of LTR retrotransposons, thus allowing to suggest that most integration events are mediated by reverse transcriptases derived from retrotransposons. Conclusion: Given the results demonstrated, it becomes evident the need for studies that characterize the ability of viral RNA to be converted into DNA forms, as well as the influence of this characteristic on the permanence of these

fragments after infection, to which proteins these genes belongs, how these genes modify and interact in viral replication and the relationship of pathogenicity and tropism associated with post-mutation protein molecular plasticity, during viral replication.

Keywords: DNA forms; Dengue vírus; Persistent infection.

Resumo

Objetivo: Estudo com abordagem teórico-experimental aplicada à expressão gênica de formas de DNA do DENV em células de mamíferos, comparada à presença de fragmentos oriundos de RNA viral descritos na literatura em células de artrópodes, experimentalmente infectadas pelo DENV. Metodologia: Trata-se de um estudo bilateral, de natureza teórico-experimental, com base na intervenção primária, realizado in vitro, sob uma abordagem técnica comparativa. Técnicas laboratoriais de expressão gênica por PCR convencional, procedimentos de verificação para análise e registros de dados da literatura foram obtidos e processados no Laboratório de Biogênese Viral, vinculado ao Setor de Arbovirologia e Febres Hemorrágicas do Instituto Evandro Chagas. Resultados: A análise experimental indicou a presença de formas de DNA de DENV-4 em células infectadas persistentemente por inoculação a 72 hpi. Como função comparativa, as células C6 / 36 apresentaram sequências de DNA viral incorporadas em regiões de retrotransposons LTR, permitindo sugerir que a maioria dos eventos de integração são mediados por transcriptases reversas derivadas de retrotransposons. Conclusão: Diante dos resultados demonstrados, fica evidente a necessidade de estudos que caracterizem a capacidade do RNA viral de ser convertido em formas de DNA, bem como a influência dessa característica na permanência desses fragmentos após a infecção, para quais proteínas esses genes pertence, como esses genes modificam e interagem na replicação viral e a relação da patogenicidade e tropismo associada à plasticidade molecular da proteína pós-mutação, durante a replicação viral.

Palavras-chave: Formas de DNA; Vírus da dengue; Infecções persistentes.

Resumen

Objetivo: Estudio con enfoque teórico-experimental aplicado a la expresión génica de formas de DNA de DENV en células de mamífero, comparado con la presencia de fragmentos provenientes de RNA viral descritos en la literatura en células de artrópodos, infectados experimentalmente por DENV. Metodología: Se trata de un estudio bilateral, de carácter teórico-experimental, con base de intervención primaria, realizado in vitro, bajo un enfoque técnico comparado. En el Laboratorio de Biogénesis Viral, vinculado al Sector de

Arbovirología y Fiebres Hemorrágicas del Instituto Evandro Chagas, se obtuvieron y procesaron técnicas de laboratorio de expresión génica por PCR convencional, procedimientos de verificación para análisis y registros de datos bibliográficos. Resultados: El análisis experimental indicó la presencia de formas de DNA de DENV-4 en células infectadas persistentemente por inoculación a 72 hpi. Como función comparativa, las células C6 / 36 presentaron secuencias de DNA viral incorporadas en regiones de retrotransposones LTR, lo que permite sugerir que la mayoría de los eventos de integración están mediados por transcriptasas inversas derivadas de retrotransposones. Conclusión: Dados los resultados demostrados, se evidencia la necesidad de estudios que caractericen la capacidad del RNA viral para convertirse en formas de DNA, así como la influencia de esta característica en la permanencia de estos fragmentos después de la infección, a qué proteínas estos genes pertenece, cómo estos genes modifican e interactúan en la replicación viral y la relación de patogenicidad y tropismo asociados con la plasticidad molecular de la proteína posmutación, durante la replicación viral.

Palabras clave: Formas de DNA; Vírus del dengue; Infecciones persistentes.

1. Introduction

Viruses whose transmission occurs through arthropods are characterized as arboviruses and these, in turn, circulate, mostly, from wild animals (vectors), causing diseases in humans and other animals of epidemiological relevance worldwide (Nag, et al, 2016; Weaver & Reisen, 2010). Some arboviruses, to the extent of the changes brought about by the conjunction between the particularities of their evolutionary apparatus and external factors, were able to remodel their enzootic amplification, making them capable of producing extensive epidemics in tropical urban centers, as observed in dengue and chikungunya (Weaver & Reisen, 2010; Bhatt, et al, 2013; Higgs, et al, 2016). In particular, dengue is a disease with a broad seasonal spectrum, dependent on environmental factors directly related to temperature, humidity and climate (Lei, et al, 2001). Presenting itself as a feverish infectious disease of acute evolution, caused by an RNA virus of the family Flaviviridae, and transmitted by a hematophagous vector (Aedes aegypti / Aedes albopictus) with high reproductive susceptibility (Brasil, 2010; Xavier, et al, 2014).

RNA viruses have the capacity for continuous modification due to genetic rearrangements, resulting in the diversity of viral genetic material, making it potentially more epidemic (Bhatt et al, 2013). This characteristic is mainly due to the errors accumulated by

RNA polymerase during replication and infection, as described by Moya et al, (2004). Allied to the genetic recombination, in association with the high mutation rates, viral adaptation and immunological environment / response, it is noted the importance of the search for the advancement of experimental studies in the process of establishing the evolution of these viruses, in order to make it susceptible the understanding of genetic mechanisms in viral RNA populations (Nag, Kramer, 2017). Like the dengue virus (DENV), most arboviruses have RNA as genetic material and, experimentally based studies, have described the presence of non-retroviral RNA virus sequences in the form of DNA in mammal and insect genomes (Goic, et al, 2013; Horie, et al, 2010; Klenerman, et al, 1997). These sequences, which may be originating either after infection by the corresponding RNA virus or by defective interfering particles that were later eliminated. Therefore, this studies do not find out whether these DNA sequences play any major role in viral replication (Nag et al, 2016).

The viral DNA forms are characterized as segments of DNA originating from viral RNA, after the infection in a cell. The presence of these DNA forms was detected in cells of uninfected mosquitoes, created in the laboratory, collected in nature and in cultures of *Aedes albopictus* cells (Nag, et al, 2016). Scientific evidence has reported the identification of specific RNA sequences in the form of DNA in infection by non-cytopathic segmented RNA virus, suggesting a specific interaction of viral RNA in the host, responsible for the conversion to DNA, determinant for persistence of the virus (Klenerman, et al, 1997). DNA sequences establish the determining potential in the generation of dsDNA during genomic transcription in the host in *Drosophila sp.* and, according to Flynt et al (2009), dsDNA was generated from the transcription of overlapping regions in opposite strands of the genome and with secondary structures. In persistent infections, Dcr2-dependent viral interfering RNAs (viRNAs) were produced through previously established regions of the viral genome with most of the viral RNAs having not been loaded on any Argonaute proteins, raising doubts as to the source of the viRNA molecules, in addition to demonstrating the regulation in viral replication in Drosophila, so that the viral DNA provided the necessary siRNAs for the establishment and maintenance of persistent infections (Goic, et al, 2013).

The originated segments, although sparsely described in the literature, are DNA forms originating from viral RNA after infection in the body, which may be related to the generation of dsRNA during cell transcription. Studies suggest the hypothesis of a direct relationship between viral persistence and conversion to viral DNA, although little is known about the influence of these fragments and which arboviruses have this characteristic (Nag, et al, 2016; Moya, et al, 2004). The extreme complexity of the interaction between the genome, host,

reservoir and vector, still little investigated, promotes a debate in the search for the mechanism of propagation of these infections.

For several arboviruses, as highlighted by DENV, due to the ineffectiveness of specific control mechanisms against the vector, strategies that monitor the molecular regulation of the agent, remain the most effective way against the spread of the infection, in order to corroborate for the understanding of vector / virus interaction mechanisms, to identify new strategies for intervention during infection and, therefore, the immune response of vertebrates at this stage, can set precedents for a new way of controlling the development of the disease (Nag, et al, 2016). Such immunological mechanisms established in the infection and produced in the arthropod are determinant in the evaluation of the presence of genomic DNA sequences related to RNA viruses (Nag, et al, 2017). Several mechanisms, such as the formation of defective interfering particles, the permanence of extracellular viral genetic material and changes in post-translation processing have been reiterated as explanations for persistent mosquito infections (Klenerman, et al, 1997).

In this perspective, the search for new information aimed at viral replication strategies combined with the analysis of the maintenance and establishment of persistent infections, has become an object of research in several regions where the dissemination of DENV has become a public health issue, from parameters related not only to viral mutation, but also to urban development and climatic susceptibility of the vector (Moya, et al, 2004). To this end, this work aims to conduct a study of theoretical and experimental approach applied to the gene expression of DNA forms in mammalian cells, compared to the presence of fragments originating from viral RNA described in the literature in arthropod cells, experimentally infected by DENV.

2. Methodology

This is a study with a bilateral strand, of a theoretical-experimental nature, primary intervention basis, carried out *in vitro*, under a comparative technical approach following the methodology followed by Nag, et al, (2017) and Pereira, et al, (2018). Laboratory techniques, analysis procedures and data records were obtained and processed at the Viral Biogenesis Laboratory, allocated to the Arbovirology and Hemorrhagic Fevers Sector of the Evandro Chagas Institute. Only isolated samples of DENv4 were used in cell cultures belonging to the collection of the section, therefore, it was not necessary to submit to the research ethics committee. After registering the generated products, the data were compared to those

described in the literature.

2.1 Cell Culture

The mammalian cells culture (VERO) were prepared in Dulbecco's Modified Eagle culture medium (DMEM). The cell culture was carried out when the monolayer confluence was approximately 70%. Subsequently, the cells were incubated in growth medium for up to 72 hours before infection.

2.2 Viral Inoculation

For viral inoculation, the growth medium was removed, the monolayers washed twice with PBS and infected with MOI 1, for this the viral samples were diluted in a medium similar to that of maintenance, but with 2% SBF, standardizing a volume. In the negative controls (uninfected cultures), up to 8 mL of medium was added. The bottles were incubated for 1 hour in an oven at 37 ° C and 5% CO2 and regularly shaken. After viral adsorption, the viral inoculums were removed and the monolayers washed up to twice with PBS to then be added 8 mL of maintenance medium, and finally incubated in an oven at 37 ° C at 5% CO2 for up to 24 hours.

2.3 Extraction and detection of DNA fragments

The DNA forms were extracted using the Spin plus 250 kit (Biopur), following the manufacturer's specifications. The detection of the amplification products was performed on a 1% agarose gel added with SYBR Safe and 100bp DNA Ladder dye and its visualization with the adjustments of magnification, focus and image capture.

2.4 Molecular tests

The PCR technique used the GoTaq Hot Start Polymerase kit (Promega), following the quantities of each reagent according to the manufacturer's manual. PCR temperature program: 94 ° C for 1 minut; 35 cycles of 94 ° C for 1 minute, 55 ° C for 1 minute, 72 ° C for 1 minute and 30 seconds and 72 ° C Hold for 7 minutes. Previously available and tested primers were used, covering the entire DENV4 genome (Table 1).

Table 1. Oligonucleotides used to search for DNA forms from the DENV4 genome.

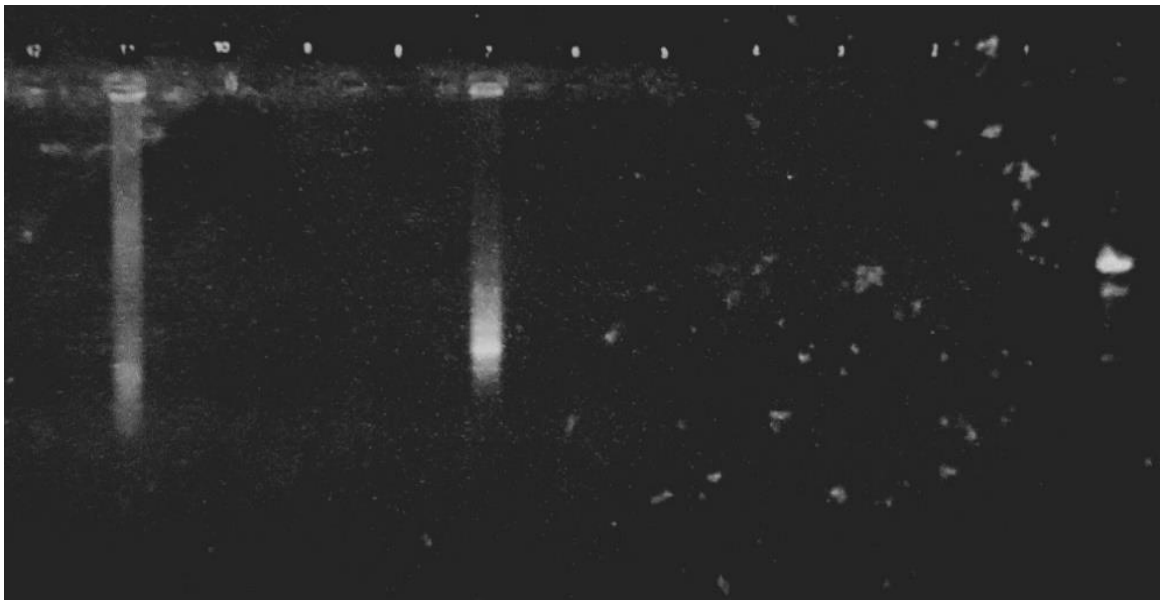
OLIGONUCLEOTIDES FOR DENV4 DNA FORMS	
Forward	**Reverse**
5’ GCTGAAACGCGAGAAGAAACC 3’	5’ GGCTCCAGCGAGGGC 3’
5’ TGATGCTGGTCGCCCCA 3’	5’ CATCCATGTGAGGCCCCC 3’
5’ TGGGGCAATGGCTGTGG 3’	5’ CCTCGACGTCCCGTATGGA 3’
5’ AGTGGTTGGGCGCGT 3’	5’ GGCTTGGGGCCCCT 3’
5’ GCACTCACACCCCCAGC 3’	5’ GGCCATGCGTTTTGCACC 3’
5’ ACGGCCGGACAGGGC 3’	5’ TGTTGGTCGTGAACATGCC 3’
5’ CCTTCCGGTGTGGCTGAG 3’	5’ GCGGCACACCGAGGTC 3’
5’ CCCCCAGGAGGGGAACTG 3’	5’ TCCAGCCCCCTGCGAG 3’
5’ CCGCAACCCCTCCCG 3’	5’ ACAGAGCCACTTCCTTGGC 3’
5’ GTGGAGACGATTGCGTGGT 3’	5’ GGCGAGCTTGGCCCT 3’
5’ AGCTGGCCCAATGGTGG 3’	5’ GGCCGCCACCACTCTC 3’
5’ GCAGCCAACGAGATGGGG 3’	5’ CCTCCTCTCCCACAGCCA 3’
5’ ACCCGGAACGTCTGGTTCT 3’	5’ TGGAGGACGCAGAGCCT 3’
5’ GCCGGTTTTGGGAACTGGT 3’	5’ TCCCAGCCTGCTGTGTCA 3’
5’ TCCGCAGTGGGAACCATCT 3’	5’ TGCGGCGCTCTGTGC 3’

Source: Authors.

3. Resullts

The results obtained during the experimental analysis indicated the presence of DNA forms in the genome of DENV-4 in cells persistently infected by inoculation at 72 hpi. The demonstrated fragments were represented by capturing the images from the data record obtained through electrophoresis on an agarose gel, indicated the sequence of POLY DV4_gp1 gene, a product of the envelope E protein (NP_740317.1) (Figure 1). The search for expression products in Nag et al (2016), were made using cells of *Aedes aegypti* (P10) infected by DENV2, maintained, extracted and expressed using the same technique described, generating bands of sizes and sequences already expected.

Figure 1. Generation of DNA forms in mammalian cells (VERO) after DENV-4 infection obtained corresponding to segments contained in structural protein E.



Source: Authors.

Similar results have already been described in the literature, as observed in Nag et al (2016), in which the authors obtained PCR-positive and negative results with genomic DNA from Aedes albopictus inoculated with P10 DENV-2 in C6 / 36 cells with a infectious clone DENV-2 pD2 / IC-30P-A as a positive control, in addition to expressions in genomic regions of DENV-2 viral RNA in forms of DNA in C6 / 36 and Aedes aegypti nucleotide P 10, indicating that the generation of the products were allowed due to model change events.

4. Discussion

Over the years, dengue has become a major public health problem in different regions of the world (Bäck, A. T., Lundkvist, 2013). Studies based on the functioning of the virus protein structure, as well as the relationship of post-infection genes in the host genome, are the most effective control mechanisms against the spread of infection and understanding of the interaction mechanisms, given that, viral proteins-related genes can be incorporated into the human cell and remain for indefinite periods, making it susceptible to reinfection (AMORIM et al, 2020; NAG et al, 2016). According to a study by Zhao et al (1987), a low immune response related to NS1 was evidenced, corresponding to a series of factors, as well as the failure to detect the polyprotein E serological response, due to its need to require an ideal processing of non-structural proteins, in addition to being produced through the

intracellular apparatus and depending on the participation of a set of structural proteins combined with the development of the viral cycle. Sohler et al, also promotes perspectives based on the theoretical basis of genetic analysis of neuro-adapted strains, revealing mutations for the viral domain that in several genes of E protein, suggested viral adaptive improvements, altering the binding in cells, increasing the replication capacity, thus suggesting the tropic multiplicity associated with neuronal cells of mice.

As a comparative function, C6/36 cells presented viral DNA sequences incorporated in regions of LTR retrotransposons, thus allowing to suggest that most integration events are mediated by reverse transcriptases derived from retrotransposons. In this way, it is possible to establish that the viral DNA forms is produced by means of former exchange events, that occurs when reverse transcriptase changes from the retroelement RNA template to viral RNA. Such possibility of exchange increases the number of copies of the heterologous RNA. The probability of generating viral DNA forms in C6/36 cells is greater than in *Aedes aegypti* cells, due to the presence of a large number of viral RNA. Consequently, C6/36 cells are likely to provide more target sequences for the PCR primers. The results obtained in VERO, corroborate the hypothesis that model changes are also observable in the mammalian organism (NAG et al, 2016).

In another perspective, Blair et al (2000), establishes in his work the promotion of studies that discuss the expression of genes of sequences belonging to specific structural proteins, related to the viral cycle, belonging to a potential host, that could associate directly to an organism, thereby interrupting replication. Studies carried out by Hapuarachchige et al, (2012), with an emphasis on assessing the spectrum of genetic characteristics of structural and non-structural proteins and their relationship in the pathogenicity of DENV, have shown a determining role during infection in mammalian cells, corroborating predominantly tropism associated with the expression of structural proteins and significant mutations in their genes. Although the impact promoted by the conformation of these structures resulting from mutation, is not yet established, as well as the impacts caused at the immunogenetic level in human cell.

Thus, it is noted that although the reactions have not generated results in significant products, it is estimated that from the products, the generation of the DNA form of the viral RNA genome occurs when the reverse transcriptase enzyme changes of the primordial model RNA for a heterologous virus RNA molecule during reverse transcription, so these results are undetectable, considering the period of days post infection (DPI) established for extraction, requiring an increase of days to inoculation of DENV (Geuking Et Al, 2009; Zhdanov, 1975).

5. Conclusion

In view of the results demonstrated, it becomes evident the need for studies that characterize the ability of viral RNA to be converted into DNA forms, as well as the influence of this characteristic on the permanence of these fragments after infection, to which proteins these genes belong, how these genes modify and interact in viral replication and the relationship of pathogenicity and tropism associated with post-mutation protein molecular plasticity during viral replication, in order to make the understanding about the DENV replication processes susceptible, as well as the virus control strategies on the cell.

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