Biochemical and Biophysical Research Communications xxx (2017) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Transcriptome analysis of *Aedes aegypti* in response to mono-infections and co-infections of dengue virus-2 and chikungunya virus

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ARTICLE INFO

Article history:
Received 25 November 2016
Received in revised form
24 January 2017
Accepted 29 January 2017
Available online xxx

Keywords:
Dengue
Chikungunya
Co-infection
Aedes
RNA-seq

ABSTRACT

Chikungunya virus (CHIKV) and Dengue virus (DENV) spread via the bite of infected Aedes mosquitoes. Both these viruses exist as co-infections in the host as well as the vector and are known to exploit their cellular machinery for their replication. While there are studies reporting the changes in Aedes transcriptome when infected with DENV and CHIKV individually, the effect both these viruses have on the mosquitoes when present as co-infections is not clearly understood. In the present study, we infected *Aedes aegypti* mosquitoes with DENV and CHIKV individually and as co-infection through nanoinjections. We performed high throughput RNA sequencing of the infected *Aedes aegypti* to understand the changes in the Aedes transcriptome during the early stages of infection, i.e., 24 h post infection and compared the transcriptome profiles during DENV and CHIKV mono-infections with that of co-infections. We identified 190 significantly regulated genes identified in CHIKV infected library, 37 genes from DENV library and 100 genes from co-infected library and they were classified into different pathways. Our study reveal that distinct pathways and transcripts are being regulated during the three types of infection states in *Aedes aegypti* mosquitoes.

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1. Introduction

Dengue virus (DENV) is an arthropod-borne single stranded RNA virus infecting around 100 million people every year and owes its complexity due to its existence as distinct serotypes (DENV1, DENV2, DENV3 and DENV4) [1,2]. The genome size of all DENV serotypes is approximately 11 kb consisting of 5′ UTR, a single polyprotein sequence which codes for three structural proteins and seven non-structural proteins and a 3′UTR [3,4]. DENV infections increase health and economic burden mainly due to complications of this infection such as dengue hemorrhagic fever and dengue shock syndrome. Currently, no drugs or vaccines are available against the virus owing to limited knowledge of DENV biology.

Chikungunya virus belongs to genus alphavirus and is transmitted by Aedes mosquitoes causing chikungunya fever in their hosts. The patients show acute fever with severe pain in joints that can last upto to several years. The first CHIKV epidemic was reported from Tanzania (East Africa) in 1952–1953 [5]. In 2005–2006, Indian Ocean outbreak caused a CHIKV epidemic in India where 1.5 million people were infected at that time. Again in 2010, a CHIKV outbreak was reported in India that affected more than one million people [6]. The CHIKV genome comprises of enveloped, single stranded positive RNA genome and exists as three genotypes, namely West African (WA), Asian (Asian) and Eastern Central South African (ECSA) strains based on the area of first isolation of that genotype. Reports show that CHIKV exists as co-infections with dengue [7], and co-morbid conditions can render this infection fatal [8].

Transmitted by *Aedes* sp. mosquitoes, these viruses exploit the cellular machinery of both the vector and host cells for its survival and expansion within the host. Upon an arboviral infection, mosquito also shows immune responses and several arthropod immunity pathways such as Toll, Imd, JAK/STAT and RNAi [9–11] were found to get impacted. Microarray analysis of flavivirus infection has shown that the genes related to transcription factor, metabolic proteins and ion-binding proteins gets up-regulated and on the other hand, pupal cuticle genes and protease shows down-regulation [12]. ERK pathway may also get induced by insulin present in blood, leading to antiviral activity [13]. Microarray analysis of blood fed mosquito infected with DENV, revealed up-regulation of genes like Rell, Toll and Spatzle, related to Toll pathway and in same study it was shown that the silencing of

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http://dx.doi.org/10.1016/j.bbrc.2017.01.162
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Please cite this article in press as: J. Shrinet, et al., Transcriptome analysis of *Aedes aegypti* in response to mono-infections and co-infections of dengue virus-2 and chikungunya virus, Biochemical and Biophysical Research Communications (2017), http://dx.doi.org/10.1016/j.bbrc.2017.01.162

MyD88 leads to the increase of viral titer in midgut [14]. Smartt and colleagues had shown the up-regulation of an unknown Toll receptor in Culex pipiens infected with WNV [15]. While these studies show the importance of immunity pathways against DENV, little is known about how these pathways behave when the mosquito is exposed to multiple pathogenic viruses at the same time.

Advances in high-throughput techniques have opened new doors to efficiently identify and analyze the molecular dynamics of virus-vector interactions and identify novel molecules that could be utilized for designing new strategies to combat the spread of these viruses. In the present study we used RNA-seq analysis to study the effect of virus mono-infection (CHIKV and DENV separately) and CHIKV/DENV co-infections on Aedes mosquitoes at their initial time points of infection, namely, 24 h post infection (24 h.p.i.). This is the first study to analyze and compare the transcriptome of Aedes during CHIKV and DENV mono- and co-infections.

2. Materials and methods

2.1. Mosquitoes rearing and nano-injections

Aedes aegypti were reared at 28 ± 1 °C and humidity was maintained at 75–80%. Sterile sucrose solution was prepared and adults were fed on the solution. 4–6 days old female mosquitoes were kept on ice and four groups of ten mosquitoes each were formed. Virus solutions containing 1×10^6 pfu/ml of chikungunya virus and dengue virus in 1xPBS were made and 49 nl of this solution was injected separately in 10 mosquitoes each. For co-infections, virus solution containing 1×10^6 pfu/ml of each of the virus was made in 1xPBS and the mixture was injected into the thorax of the third group of 10 mosquitoes. Additionally, one more group was injected with 49 nl of 1× PBS solution as used as the mock group and served as a control. Mosquitoes were allowed to recover and were collected after 24 h post injection. Nanoinjections of the infected and PBS groups were performed for a minimum of three times. The mosquitoes were then pooled group-wise and stored until further analysis.

2.2. Sample preparation

The collected pools of mosquitoes from each batch were stored in Trizol and processed separately. The mosquitoes were homogenized properly and the mixture was centrifuged and supernatant was collected. 250 μl of chloroform was added to the supernatant and kept at room temperature for 5 min. The solution was again centrifuged at 13000 rpm for 10 min at 4° C. Supernatant was collected and 500 μl of chilled isopropanol was added to it, gently mixed and kept for 5 min. It was again centrifuged at 13000 rpm for 10 min at 4° C. The pellet was collected and washed twice with 500 μl of 70% ethanol and the solution was centrifuged at 7000 rpm for 10 min. The pellet was dried at room temperature for 5–7 min and 50 μl of DEPC treated water was added to it and the mixture was kept at room temperature for another 5–10 min with gentle tapping in between. Finally the RNA was checked on 1% TBE gel and NanoDrop reading was also recorded for each of the sample separately.

2.3. Preparation of library and sequencings

Total RNA was extracted from each group of collected *Ae. aegypti* mosquitoes namely, Mock mosquitoes (hereafter MM), Chikungunya infected mosquitoes (CM), Dengue infected mosquitoes (DM) and CHIKV/DENV Co-infected mosquitoes (CD) and Paired-End RNA-seq libraries were generated. The construction of RNA-Seq library and sequencing of the libraries were performed by commercial service providers Sandor Life Sciences Pvt. Ltd., Hyderabad, Telangana, India. mRNA enrichment from total RNA was performed using Oligotex mRNA midi prep kit (QIAGEN, Germany). 2 μg of RNA was fragmented and first strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Life Technologies, Inc.) and random primers. After second strand cDNA synthesis the fragments were A-tailed. RNA Sample Preparation Kit (Illumina, Inc.) was used to construct cDNA libraries of the, according to manufacturer's instructions. The Paired-End RNA-Seq libraries were sequenced using Illumina NextSeq 500 platform for generating 2 × 76 bp sequencing reads.

2.4. Transcriptome assembly and read mapping

A workflow of the transcriptome analysis is shown in Fig. 1. The adapter sequences from the reads of all the libraries were trimmed using fastx toolkit and quality check was performed on the trimmed sequences using FastQC tool [16]. The high quality reads with quality score ≥20 were retained for further analysis. High quality reads were mapped on *Aedes aegypti* genome downloaded from VectorBase [17] using TopHat [18] tool which uses Bowtie [19] to align the RNA-seq reads and it breaks the unaligned reads into small fragments for realigning them with genome. Cufflinks package was used for further analysis like transcript assembly and comparing transcript assemblies to annotation [18]. The annotations of identified transcripts were fetched from VectorBase database using BioMart tool.

2.5. Differential expression and statistical analysis

A Bioconductor package edgeR was used to perform differential expression analysis of the different libraries [20]. edgeR performs normalization of the read counts using Trimmed mean of M values (TMM) approach. Identification of differentially expressed genes were performed by comparing the libraries against mock, i.e., CM vs MM, DM vs MM and CD vs MM. Dispersion value of 0.1 and p-value $<= 0.05$ were taken while performing edgeR analysis for identifying the significantly regulated transcripts.

Pathway enrichment analyses were also carried out for the identified significantly expressed genes. The Ensembl Gene ID of the differentially expressed genes were extracted from VectorBase using BioMart tool and submitted to KOBAS 2.0 web server for identification of significant pathways with p-value ≤ 0.05 [21]. Statistical analysis was performed by selecting Hypergeometric test/Fisher's exact test as statistical method and Benjamin and Hochberg method (1995) was used for FDR correction.

3. Results

3.1. Data analysis and mapping of RNA-seq data

To understand the early molecular changes in Aedes during DENV and CHIKV development as mono-infection and co-infection in mosquitoes, we delivered both these viruses using nano-injections in the thoracic region of mosquitoes. After 24 h, ten mosquitoes from each group were taken, total RNA was isolated and paired-end RNA sequencing was performed for each group. We generated a total of 1.42×10^8 raw reads from all the libraries taken together. The adapters were removed and reads were further filtered and trimmed on the basis of quality scores by taking quality score threshold as 20 generating a total of 1.30×10^8 reads. Out of these reads, 4.16×10^7 reads were from MM, 3.31×10^7 reads belongs to mosquitoes infected with chikungunya virus (CM), DM constitutes 2.13×10^7 reads and 3.20×10^7 reads were from CD library. Mapping of the reads from each library to *Aedes aegypti*

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