Discovery of Mosquito Saliva MicroRNAs during CHIKV Infection

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Abstract

Mosquito borne pathogens are transmitted to humans via saliva during blood feeding. Mosquito saliva is a complex concoction of many secretory factors that modulate the feeding foci to enhance pathogen infection and establishment. Multiple salivary proteins/factors have been identified/characterized that enhance pathogen infection. Here, we describe, for the first time, the identification of exogenous microRNAs from mosquito saliva. MicroRNAs are short, 18–24 nucleotide, non-coding RNAs that regulate gene expression, and are generally intracellular. However, circulating miRNAs have been described from serum and saliva of humans. Exogenous miRNAs have not been reported from hematophagous arthropod saliva. We sought to identify miRNAs in the mosquito saliva and their role in Chikungunya virus (CHIKV) infection. Next generation sequencing was utilized to identify 103 exogenous miRNAs in mosquito saliva of which 31 miRNAs were previously unidentified and were designated novel. Several miRNAs that we have identified are expressed only in the CHIKV infected mosquitoes. Five of the saliva miRNAs were tested for their potential to regulated CHIKV infection, and our results demonstrate their functional role in the transmission and establishment of infection during blood feeding on the host.

Author Summary

Mosquito saliva contains a complex repertoire of bioactive factors that are secreted into blood feeding site, the skin. Infected mosquitoes transmit pathogens to the host during feeding via saliva. The bioactive factors in mosquito saliva are responsible for modulating host hemostasis, immune defenses and pain/itch responses, and have been implicated to enhance pathogen infection and establishment in the host. In our efforts to identify and characterize salivary immunomodulators that enhance Chikungunya virus (CHIKV) transmission, we have discovered, for the first time, exogenous microRNA in mosquito saliva. MicroRNAs (miRNAs) are short, 18–24 nucleotide, non-coding RNAs that regulate gene expression. Short non-coding RNAs were extracted from the saliva of Chikungunya infected mosquitoes.
virus (CHIKV) infected and uninfected Aedes aegypti and Aedes albopictus saliva, and subjected to Illumina next generation sequencing. Bioinformatic analysis revealed the presence of miRNAs in the mosquito saliva. We have also identified several novel miRNAs that are expressed only during CHIKV infection. Though the functional roles of these miRNAs are yet to be established, our in-vitro data from testing 5 miRNAs demonstrate their role in the regulation of CHIKV infection. These miRNAs may play an important role in regulating the establishment of CHIKV infection in the mammalian host during blood feeding.

Introduction
Mosquitoes are a significant public health concern due to their ability to transmit a variety of emerging and reemerging arboviruses [1,2]. Chikungunya virus (CHIKV) is an excellent example of globalization of a mosquito borne disease, as evident from the CHIKV epidemics in the past seven years [3,4]. Chikungunya virus is an Alphavirus belonging to the Togaviridae family and is transmitted predominantly by Aedes aegypti and Aedes albopictus (www.cdc.gov/ncidod/dvbid/Chikungunya). Aedes aegypti and Ae. albopictus transmit CHIKV during blood meal acquisition, along with the saliva the mosquitoes inject into the skin. The complex repertoire of secretory proteins/factors in the mosquito saliva creates an immunologically compromised micro-environment that can have a profound effect on the transmission efficiency, pathogen establishment, and disease development [5–7]. The presence of Ae.aegypti saliva causes a differential host immune response to CHIKV infections in mice [6], suppresses recruitment of T cells to the initial bite site thus enhancing West Nile virus dissemination [8], suppresses antimicrobial peptides and IFNs thus enhancing Dengue virus (DENV) infection in human keratinocytes [9] and modulates Rift Valley Fever virus pathogenicity in mice [10]. To that end, several saliva proteins have been isolated that are facilitators of mosquito feeding, modulators of skin immunity and regulators of virus transmission and dissemination in the vertebrate host [11]. For example, the aegyptin protein isolated from Ae.aegypti saliva aids in blood feeding [12]. Another isolated putative 34 kDa protein modulates DENV infection in human keratinocytes via immunomodulation [13] and serine proteases in Ae.aegypti saliva facilitate DENV dissemination in mice [11]. These studies provide important information about the complex roles of salivary proteins in virus-host interactions however, other components of saliva and their functions have not been identified or characterized.

MicroRNAs (miRNAs) are short 18–24 nucleotide non-coding RNAs that regulate gene expression post-transcriptionally by binding to complementary regions mainly in the 3’ UTRs of targeted messenger RNAs. MicroRNA expression patterns have been profiled in mosquitoes of medical importance such as Anopheles gambiae [14], Anopheles stephensi [15], Ae.aegypti [16], Ae. albopictus [17], Culex quinquefasciatus [18] and Anopheles anthropophagus [19]. Functional studies of these mosquito miRNAs have demonstrated their role in blood digestion and egg development in Ae. aegypti [20], blood-meal induced miRNA expression for regulation of immune genes in Ae. aegypti [21] and Ae. albopictus [22], altered patterns of expression in An. stephensi post-blood feeding [23] and growth-stage specific expression in An. anthropophagus [19]. These miRNA expression profiles are altered in mosquitoes infected with parasites. For instance, the obligate endosymbiont, Wolbachia pipientis, regulates specific miRNA levels for maintenance of its life cycle in Ae.aegypti mosquitoes [24,25]. MicroRNA levels were also manipulated in An.stephensi [23] and An. gambiae [14] infected with Plasmodium and in Ae.aegypti infected with Dengue 2 [26]
While miRNAs have been detected and profiled from mosquito cell lines and mosquitoes, miRNA profiles in mosquito saliva have not been investigated. In the present study, we sought to detect and identify miRNAs in the saliva of *Ae.aegypti* and *Ae. albopictus* mosquitoes via deep sequencing. Furthermore, to investigate the effect of CHIKV infection on saliva miRNA expression profiles, deep sequencing was also performed on CHIKV-infected *Ae.aegypti* and *Ae.albopictus* saliva. A total of 103 mature miRNAs were discovered in *Ae.aegypti* and *Ae.albopictus* saliva. Seventy-two of the detected miRNAs aligned with previously identified miRNAs while 31 were potential novel miRNAs. Furthermore, 59 and 30 known miRNAs were upregulated in *Ae.aegypti* and *Ae. albopictus* CHIKV-infected saliva respectively indicating the possible functional importance of these miRNAs in CHIKV dissemination and transmission in the host.

**Methods**

**Cells and viruses**

African green monkey kidney (Vero) cells were maintained with Dulbecco’s Modified Eagle Medium (DMEM; Gibco, Carlsbad, CA) and baby hamster kidney (BHK-21) cells were maintained with Modified Eagle’s Medium (MEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA) and 5% penicillin/streptomycin (P/S; 100U/mL/100μg/mL, Gibco, Carlsbad, CA) at 37°C with 5% CO₂. The *Aedes albopictus* (C6/ 36) cell line was maintained in Leibowitz’s media (Invitrogen) supplemented with 10% FBS and 5% P/S at 28°C without CO₂. The *Aedes Ae.aegypti* (AAG-2) cell line was maintained in Schneider’s Insect Cell Media (Invitrogen) supplemented with 10% FBS and 5% P/S at 28°C without CO₂. The infectious clone, CHIKV-LR 5₀GFP (CHIKV), used in this study has been described and characterized previously [27], and was provided by Dr. Stephen Higgs.

**Mosquitoes**

The *Aedes aegypti* (Higgs White eye) strain and the *Aedes albopictus* (La Reunion) strain are well characterized and competent vectors for CHIKV and CHIK-LR 5₀GFP viruses [27]. Mosquitoes were reared as previously described [16] within the UTMB insectary services core facility. Both species of mosquitoes were maintained at 28°C at a 14:10 hour (L:D) photoperiod with 10% sucrose solution provided *ad libitum*. Three to five day old females were used for all intrathoracic inoculations.

**Intrathoracic inoculations**

Three to 5 day-old *Ae.aegypti* and *Ae. albopictus* mosquitoes were cold-anesthetized and intrathoracically inoculated with an approximately 0.1 μL inoculum of CHIKV-LR 5₀GFP: 4.6 TCID₅₀/mL. One hundred mosquitoes were inoculated per species after which inoculated mosquitoes were placed in 1 pint cartons in a 28°C incubator with 10% sucrose supplied *ad libitum* and a 14:10 hour (L:D) photoperiod. After 10 days post-infection (d.p.i), 50 infected and 50 uninfected mosquitoes were collected for each species, cold-anesthetized and saliva was collected. Briefly, saliva was collected by inserting each mosquito proboscis in a capillary tube with approximately 10μL of immersion oil and letting each mosquito salivate for 30 minutes at room temperature. Saliva were pooled according to infection status and species of mosquito, mixed with 250μL of DMEM and stored at −80°C until further processing.

**RNA extractions**

The miRNeasy Kit (Qiagen, Valencia, CA) was used for extraction of microRNAs from the mosquito saliva. Briefly, 250μL of Trizol LS (Invitrogen, Carlsbad, CA) was added to the pooled
mosquito saliva samples for virus inactivation and incubated overnight at −20°C. After 24 hours post-inactivation, RNA samples were thawed and 150μL of chloroform was added to each tube and shaken vigorously for 30 seconds. The samples were centrifuged for 15 minutes at 10000 × g at 4°C after which the clear, top layer was transferred to a new tube for total RNA and miRNA extraction using the Qiagen RNeasy extraction kit and Qiagen microRNA extraction kit respectively.

**Next generation sequencing**

The Illumina TruSeq SmallRNA kit was used to prepare libraries of the microRNA samples. Briefly, short unique adapters were ligated to the 5' and 3' ends of short RNAs. Reverse-transcriptase and PCR were used to add the full length adapters required for Illumina sequencing, followed by gel purification of the correct size templates. The samples were tracked using “index tags” incorporated into the adapters. Library quality was evaluated using an Agilent DNA-1000 chip on an Agilent 2100 Bioanalyzer. Quantification of library DNA templates was performed using qPCR and a known-size reference standard.

**Sequence analysis**

Cluster formation of the library DNA templates was performed using the TruSeq PE Cluster Kit v3 (Illumina) and the Illumina cBot workstation using conditions recommended by the manufacturer. Template input was adjusted to obtain a cluster density of 700–850 K/mm². 50 base sequencing by synthesis was performed using TruSeq SBS kit v3 (Illumina) on an Illumina HiSeq 1000 using protocols defined by the manufacturer.

**Data analysis**

The miRDeep2 software package [28] identified potential miRNA precursors by scanning for pileups of short reads in the genome alignment data. The region surrounding the pileup was excised computationally and analyzed for miRNA features. The structure of the potential precursor RNA was analyzed by RNAfold to determine the predicted secondary structure of the region and that structure was compared to typical miRNA precursor structures. If a likely structure was found, reads mapped to the precursor were counted and analyzed for the presence of mature and star miRNA sequences and then compared to the level of background sequences. The miRDeep2 algorithm used these results to score the likelihood that the predicted miRNA was real. The number of reads for each unique sequence was tracked. Following the miRDeep2 workflow the microRNAs were then compared against known microRNAs from the miRBase database (Version 20) with Aedes aegypti (AaegL1) as the reference species and Anopheles gambiae (AgamP3) as a related species. As the Ae.albopictus genome sequence was unavailable and miRNAs are highly conserved between species, reads from Ae.albopictus saliva were compared to known Ae. aegypti and An. gambiae miRNAs from miRBase database (Version 20). Novel microRNAs were identified by mapping the reads to the Ae. aegypti genome (AaegL2 from VectorBase VB-2014-02). Finally a table of known and potentially novel miRNAs was output with mapped read counts for each. Relative abundance of miRNAs in CHIKV infected samples were calculated by normalizing the data by tags per million (TPM) reads of total RNA as described previously [29].

**MicroRNA inhibition assay**

MicroRNA inhibitors were designed based on the sequences of the following select microRNAs, aae-mir-12, aae-mir-125, aae-mir184, aar-mir-375, aae-mir-2490 and a control.
inhibitor with random sequence, Scramble, that was designed based on a previous study [30]. All miRNA inhibitors (MIR-12, MIR-125, MIR-184, MIR375 and MIR-2490) were synthesized by Integrated DNA Technologies®. The microRNAs that were chosen for this miRNA inhibition study were selected based on relative abundance levels of CHIKV-infected saliva, as well as, previous reports indicating their importance in modulating DENV and Wolbachia replication [21,24,31,32]. However, they have not been studied in the context of CHIKV replication. Additionally, these miRNAs have been identified and characterized in both AAG-2 and C6/36 mosquito cell lines [21,24,25,32,33]. Baby hamster kidney cells were used for this study as they are a fibroblast cell line and CHIKV targets and replicates in fibroblast cells in a natural infection [34,35]. The cell lines, AAG-2, BHK-21 and C6/36 cells, were grown to confluency and transfected in triplicate with 100 nanograms of each miRNA inhibitor via Cellfectin transfection reagent. As a control, cells were mock transfected without template. Cells were re-transfected at 48 hours post-transfection and were infected with CHIKV at a multiplicity of infection of 0.01 at 72 hours after initial transfection. As a control, mock transfected cells were also infected with CHIKV. Daily timepoints of 50 μL were collected from each replicate until 72 hours post-infection, added to 450 μL of diluent and stored at −80°C until further processing. A standard plaque assay on Vero cells was used to determine CHIKV titer at each time point as previously described [36].

Statistical analysis

A 2-tailed student’s T-test (α 0.05) was used to analyze the significance of viral titer differences in the miRNA inhibition assay at each time point.

Results

Small RNA sequencing of Aedes spp. saliva

Small RNAs were extracted from the saliva of uninfected Ae.aegypti and Ae.albopictus mosquitoes and Ae.aegypti and Ae. albopictus mosquitoes infected with CHIKV. These small RNAs were then sequenced via Illumina-based high-throughput sequencing in order to identify small non-coding RNAs. A total of 14 × 10⁶ small RNAs were detected in Ae.aegypti saliva with a predominant size distribution of 18–33 nucleotides (nt) (Fig. 1A). Out of these, 18–24mers represented 56% of the library where 18mers represented a higher percentage of the library at 19% (Fig. 1A). After these RNAs were aligned with the Ae.aegypti genome, 43% of the Ae. aegypti library was composed of 18–24mers with 22mers exhibiting the highest frequency of reads (Fig. 1B). In comparison, small RNA sequencing of Ae.albopictus saliva, detected 3 × 10⁶ small RNAs and demonstrated a larger size range of 18–40 nts out of which 21% were represented by 18–24mers (Fig. 1C). Ae.albopictus saliva small miRNAs were matched to known Ae. aegypti and An.gambiae miRNAs and demonstrated a 48.8% representation of 18–24mers with 22mers having the highest frequency of reads (Fig. 1D).

In order to confirm that the detected 18–24mers were indeed mature miRNAs, miRDeep2 software was utilized to identify potential miRNAs based on nucleotide length, star sequence, stem-loop structural folding and sequence homology to already established reference genomes. Novel miRNAs were also identified using the criteria mentioned above. The stem-loop structures, the star sequence and premature miRNA sequence are shown for a select few novel miRNAs including miR-aae-249, miR-aae-23, miR-aal-43b and miR-aal-5. (S1 Fig.). Thirty-two percent of the detected miRNAs aligned to insect and mammal-specific miRNAs. Twenty-five percent were insect-specific and 11% were mosquito-specific miRNAs. Notably, 31% of the detected saliva miRNAs did not align with any known Ae.aegypti and An.gambiae miRNAs and were therefore designated as novel Ae.aegypti and Ae.albopictus miRNAs. Taken together
these data provide strong evidence for the presence of mature insect miRNAs in *Ae.aegypti* and *Ae.albopictus* saliva.

### Identification of *Ae.aegypti* saliva miRNAs

After aligning the sequencing reads from *Ae.aegypti* saliva to the *Ae.aegypti* miRNA database, 72 distinct known miRNAs were identified in both uninfected and CHIKV-infected mosquito saliva (Table 1). In uninfected *Ae.aegypti* saliva, a total of 298283 reads were obtained with 283197 reads aligning with known miRNAs and 15086 reads that were novel miRNAs. In comparison, the total read count in CHIKV-infected *Ae.aegypti* saliva was 305894 reads with 251277 known miRNA reads and 54617 novel miRNA reads. The highest expressing miRNA in uninfected *Ae.aegypti* saliva was aae-mir-281-2-5p at 80151 reads. The other highly expressed miRNAs in uninfected *Ae.aegypti* saliva were aae-mir-281 (56394), aae-mir-2940 (25307),

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**Figure 1. Identification of microRNAs in *Aedes* spp. saliva.** *Aedes aegypti* and *Aedes albopictus* were intra-thoracically infected with Chikungunya virus. At 10 days post infection, saliva was collected from both infected and uninfected mosquitoes. Small RNAs were extracted from the saliva and subjected to deep sequencing, small RNA libraries were created and mapped to *Ae.aegypti* and *An.gambiae* miRNA databases. Figure a) size distribution and percentages of small RNAs in *Ae.aegypti* saliva, b) percentages of 18–24 nucleotide microRNAs in *Ae.aegypti* saliva library, c) size distribution and percentages of small RNAs in *Ae. albopictus* saliva, d) percentages of 18–24 nucleotide microRNAs in *Ae. albopictus* saliva library.

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Table 1. Read counts of individual microRNAs detected in uninfected and CHIKV-infected *Ae.aegypti* saliva.

| MicroRNA          | Read counts | Difference between infected and uninfected | Fold Difference |
|-------------------|-------------|---------------------------------------------|-----------------|
|                   | Infected    | Uninfected                                 | Difference      |                |
| aae-mir-8         | 50004       | 47613                                       | 2391            | 1.2            |
| aae-mir-2940      | 21514       | 25307                                       | -3793           | 1.0            |
| aae-mir-263a      | 20584       | 9084                                        | 11500           | 2.6            |
| aae-bantam        | 18002       | 9969                                        | 8033            | 2.0            |
| aae-mir-125       | 15735       | 5863                                        | 9872            | 3.0            |
| aae-mir-281       | 5833        | 56394                                       | -50561          | 0.1            |
| aae-mir-281-2-5p  | 9818        | 80151                                       | -70333          | 0.1            |
| aae-mir-100       | 13160       | 4309                                        | 8851            | 3.4            |
| aae-mir-14        | 12958       | 5389                                        | 7569            | 2.7            |
| aae-mir-285       | 10006       | 31                                          | 9975            | 363.8          |
| aae-mir-276-1     | 9384        | 2584                                        | 6800            | 4.1            |
| aae-mir-276-2     | 9291        | 2570                                        | 6721            | 4.1            |
| aae-mir-317-1     | 6263        | 3367                                        | 2896            | 2.1            |
| aae-mir-317-2     | 6263        | 3367                                        | 2896            | 2.1            |
| aae-mir-184       | 5080        | 10105                                       | -5025           | 0.6            |
| aae-mir-12        | 4288        | 537                                         | 3751            | 9.0            |
| aae-mir-277       | 3210        | 798                                         | 2412            | 4.5            |
| aae-mir-10        | 2815        | 2035                                        | 780             | 1.6            |
| aae-mir-279       | 2315        | 587                                         | 1728            | 4.4            |
| aae-mir-2a        | 1695        | 580                                         | 1115            | 3.3            |
| aae-mir-11        | 1650        | 2309                                        | -659            | 0.8            |
| aae-mir-1891-2    | 1576        | 1078                                        | 498             | 1.6            |
| aae-mir-1891-1    | 1576        | 1078                                        | 498             | 1.6            |
| aae-mir-1889      | 1195        | 298                                         | 897             | 4.5            |
| aae-mir-2c        | 1133        | 313                                         | 820             | 4.1            |
| aae-mir-210       | 1080        | 28                                          | 1052            | 43.5           |
| aae-mir-34        | 1036        | 790                                         | 246             | 1.5            |
| aae-mir-2b        | 905         | 219                                         | 686             | 4.7            |
| aae-mir-92a       | 891         | 227                                         | 664             | 4.4            |
| aae-mir-306       | 866         | 419                                         | 447             | 2.3            |
| aae-mir-927       | 771         | 116                                         | 655             | 7.5            |
| aae-mir-71        | 764         | 352                                         | 412             | 2.4            |
| aae-mir-275       | 735         | 271                                         | 464             | 3.1            |
| aae-mir-92b       | 700         | 430                                         | 270             | 1.8            |
| aae-mir-996       | 693         | 169                                         | 524             | 4.6            |
| aae-let-7         | 665         | 616                                         | 49              | 1.22           |
| aae-mir-305       | 575         | 111                                         | 464             | 5.8            |
| aae-mir-970       | 543         | 259                                         | 284             | 2.4            |
| aae-mir-957       | 533         | 43                                          | 490             | 14.0           |
| aae-mir-999       | 478         | 144                                         | 334             | 3.7            |
| aae-mir-252       | 410         | 340                                         | 70              | 1.4            |
| aae-mir-13        | 409         | 67                                          | 342             | 6.9            |
| aae-mir-9c        | 332         | 299                                         | 33              | 1.3            |
| aae-mir-980       | 330         | 18                                          | 312             | 20.7           |
| aae-mir-133       | 277         | 11                                          | 266             | 28.4           |

(Continued)
Similarly, the highest expressing miRNAs in CHIKV-infected *Ae. aegypti* saliva were aae-mir-8 (50004), aae-mir-2940 (21514), aae-mir-263a (20584), aae-mir-bantam (18002), aae-mir-125 (15735), aae-mir-100 (13160), aae-mir-14 (12958) and aae-mir-285 (10006) (Table 1).

**Table 1.** (Continued)

| MicroRNA      | Read counts | Difference between infected and uninfected |
|---------------|-------------|------------------------------------------|
|               | Infected | Uninfected | Difference | Fold Difference |
| aae-mir-1000-2| 226      | 2          | 224        | 127.4           |
| aae-mir-1000-1| 226      | 2          | 224        | 127.4           |
| aae-mir-998   | 211      | 140        | 71         | 1.7             |
| aae-mir-190   | 190      | 36         | 154        | 5.9             |
| aae-mir-308   | 172      | 44         | 128        | 4.4             |
| aae-mir-307   | 161      | 0          | 161        | 161.0           |
| aae-mir-315   | 146      | 10         | 136        | 16.5            |
| aae-mir-263b  | 133      | 7          | 126        | 21.4            |
| aae-mir-9a-2  | 112      | 67         | 45         | 1.9             |
| aae-mir-9a-1  | 112      | 67         | 45         | 1.9             |
| aae-mir-1890  | 109      | 33         | 76         | 3.7             |
| aae-mir-932   | 109      | 50         | 59         | 2.5             |
| aae-mir-2941-2| 104      | 177        | –73        | 0.7             |
| aae-mir-2941-1| 100      | 168        | –68        | 0.7             |
| aae-mir-87    | 98       | 34         | 64         | 3.2             |
| aae-mir-2945  | 96       | 21         | 75         | 5.2             |
| aae-mir-278   | 91       | 80         | 11         | 1.3             |
| aae-mir-33    | 86       | 11         | 75         | 8.8             |
| aae-mir-31    | 74       | 11         | 63         | 7.6             |
| aae-mir-981   | 73       | 10         | 63         | 8.2             |
| aae-mir-989   | 72       | 616        | –544       | 0.1             |
| aae-mir-2946  | 68       | 234        | –166       | 0.3             |
| aae-mir-375   | 54       | 189        | –135       | 0.3             |
| aae-mir-283   | 49       | 225        | –176       | 0.2             |
| aae-mir-9b    | 34       | 64         | –30        | 0.6             |
| aae-mir-1174  | 18       | 158        | –140       | 0.1             |
| aae-mir-1175  | 12       | 96         | –84        | 0.1             |

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**Detection of novel *Ae.aegypti* saliva miRNAs**

Thirty-one novel mature miRNAs were detected from *Ae.aegypti* saliva after the predicted miRNAs were compared to the *Ae.aegypti* miRNA database and AaegL2 (Table 2). The highest expressed novel miRNA was aae-mir-143 with a count of 4275 and with a seed sequence match to aga-mir-14 (Table 2). Aae-mir-249, aae-mir-80 and aae-mir-5 were also highly expressed novel miRNAs in uninfected *Ae.aegypti* saliva with counts of 5385, 3773 and 2566 respectively (Table 2).

**Identification of *Ae.albopictus* saliva miRNAs**

A total of 43 miRNAs were identified in *Ae.albopictus* saliva. In uninfected *Ae.albopictus* saliva, a total of 12075 reads were obtained with 9180 reads aligning with known miRNAs and 4741
reads that were novel miRNAs (Table 3). In contrast, the total read count was 2-fold higher in CHIKV-infected *Ae.albopictus* saliva with a total count of 32593 reads with 16050 known miRNA reads and 16543 novel miRNA reads. Twenty-eight known miRNAs were identified in *Ae.albopictus* saliva (Table 3). The highest expressed miRNA in uninfected *Ae.albopictus* saliva was aae-mir-8 with a count of 12874 followed by aae-mir-2940 (2574), aae-mir-bantam (2127) and aae-mir-125 (2132) (Table 3). Highest read counts in CHIKV-infected *Ae.albopictus* saliva were from aae-mir-125 (4333), aae-mir-263a (4293), aae-mir-8 (2609), aae-mir-100 (2255) (Table 3). With the exception of aae-mir-8, these miRNAs were upregulated at least 1.3-fold or higher in comparison with uninfected saliva (Table 3).

Identification of novel miRNAs in *Ae.albopictus* saliva

Twenty-four novel, mature miRNAs were detected in *Ae.albopictus* saliva (Table 4). The highest expressing miRNA in uninfected *Ae.albopictus* saliva was aal-mir-43b which had a read

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**Table 2.** Read counts of individual novel microRNAs detected in uninfected and CHIKV-infected *Ae.aegypti* saliva.

| Assigned Name | Consensus sequence | Read Counts | Difference between infected and uninfected |
|---------------|--------------------|-------------|--------------------------------------------|
|               |                    | Infected    | Uninfected | Difference | Fold Difference |
| aae-mir-143   | aacccguagauccgaacuugug | 13129       | 4275       | 8854       | 0.8            |
| aae-mir-249   | uagcuuccuuuucuccuucu | 12951       | 5385       | 7566       | 0.7            |
| aae-mir-5     | uaggaacucucauccaugcuucu | 9219       | 2566       | 6653       | 1.0            |
| aae-mir-229   | ucauuaagacacgcggcaau | 544        | 259        | 285        | 0.6            |
| aae-mir-778   | uuggcccuucaacaccaguugu | 278        | 11         | 267        | 7.0             |
| aae-mir-620   | auuagaugggauaauuaguuu | 51         | 3          | 48         | 4.7             |
| aae-mir-3069  | uuguuucguuuggcucgagu | 54         | 188        | –134       | 0.1             |
| aae-mir-744   | cauccacucuaguuccuguucu | 1451      | 1783       | –332       | 0.2             |
| aae-mir-115   | uugauugagcuagugagguac | 71         | 616        | –545       | 0.0             |
| aae-mir-23    | uagcaccuauccaaauacaguac | 10021     | 0          | 10021      | 10021.0         |
| aae-mir-576   | ggggaugucucugcugagguag | 2033      | 0          | 2033       | 2033.0          |
| aae-mir-320   | uuucggauauguuuuauaaccu | 1262      | 0          | 1262       | 1262.0          |
| aae-mir-214   | uuucggacgacgcctcca | 606        | 433        | 173        | 0.4             |
| aae-mir-402   | uuucggacgacgcctcca | 606        | 433        | 173        | 0.4             |
| aae-mir-65    | ugcacagcuacuaggggaugac | 397       | 0          | 397        | 397.0           |
| aae-mir-341   | gcggagucrgcggagcu | 294         | 0          | 294        | 294.0           |
| aae-mir-3     | ggguagcaggguuuggauuuc | 250       | 0          | 250        | 250.0           |
| aae-mir-3798  | aauauugcuugucacagcag | 226       | 0          | 226        | 226.0           |
| aae-mir-187   | aauauuucugucugacagcag | 226      | 0          | 226        | 160.0           |
| aae-mir-242   | caucugagcucgccuga | 160         | 0          | 160        | 139.0           |
| aae-mir-309   | guaggccgagcgaaacuacugc | 139    | 21         | 118        | 1.8             |
| aae-mir-210   | uuaccaauccagcaugcc | 129         | 0          | 129        | 129.0           |
| aae-mir-1571  | gaggguccgguuauacu | 88          | 0          | 88         | 88.0             |
| aae-mir-1247  | guugacuuccaccagcgu | 81          | 12         | 69         | 1.9             |
| aae-mir-40    | uuugguuauuaauucggc | 81          | 0          | 81         | 81.0             |
| aae-mir-843a  | gcccugugccgcucgcuc | 74          | 0          | 74         | 81.0             |
| aae-mir-117   | uacgaaucugcuagggac | 73          | 0          | 73         | 74.0             |
| aae-mir-360   | guagcaaaauucuggu | 67          | 0          | 67         | 73.0             |
| aae-mir-359   | guacugacugagcag | 56          | 0          | 56         | 67.0             |
| aae-mir-80    | auuucucugucucaccca | 0          | 3773       | –3773      | 0.0             |
| aae-mir-109   | aucacugcggguacacca | 0          | 227        | –227       | 0.0             |

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Table 3. Read counts of individual microRNAs detected in uninfected and CHIKV-infected *Ae.albopictus* saliva.

| MicroRNAs | Read Counts | Difference between Infected and Uninfected | Fold difference |
|-----------|-------------|---------------------------------------------|-----------------|
|           | Infected    | Uninfected                                  | Difference      |
| aae-mir-125 | 4333        | 2132                                        | 2201            | 2.2 |
| aae-mir-263a | 4293        | 1343                                        | 2950            | 3.4 |
| aae-mir-8 | 2609        | 12874                                       | −10265          | 0.2 |
| aae-mir-184 | 2332        | 1885                                        | 447             | 1.3 |
| aae-mir-100 | 2255        | 1204                                        | 1051            | 2.0 |
| aae-mir-2940 | 1923       | 2574                                        | −651            | 0.8 |
| aae-mir-281 | 377         | 210                                         | 167             | 1.9 |
| aae-mir-281-2-5p | 752   | 292                                         | 460             | 2.7 |
| aae-bantam | 1116        | 2127                                        | −1011           | 0.6 |
| aae-mir-276-1 | 1046      | 482                                         | 564             | 2.3 |
| aae-mir-276-2 | 1038       | 482                                         | 556             | 2.3 |
| aae-mir-14 | 1032        | 731                                         | 301             | 1.5 |
| aae-mir-10 | 887         | 153                                         | 734             | 6.2 |
| aae-mir-927 | 795         | 151                                         | 644             | 5.6 |
| aae-mir-317-1 | 632        | 844                                         | −212            | 0.8 |
| aae-mir-317-2 | 632        | 844                                         | −212            | 0.8 |
| aae-mir-277 | 430         | 212                                         | 218             | 2.2 |
| aae-let-7 | 423         | 195                                         | 228             | 2.3 |
| aae-mir-999 | 409         | 146                                         | 263             | 3.0 |
| aae-mir-11 | 385         | 381                                         | 4               | 1.1 |
| aae-mir-957 | 361         | 107                                         | 254             | 3.6 |
| aae-mir-34 | 339         | 619                                         | −280            | 0.6 |
| aae-mir-92b | 304         | 45                                          | 259             | 7.2 |
| aae-mir-275 | 276         | 109                                         | 167             | 2.7 |
| aae-mir-315 | 273         | 16                                          | 257             | 18.1 |
| aae-mir-2a | 212         | 171                                         | 41              | 1.3 |
| aae-mir-2c | 182         | 143                                         | 39              | 1.4 |
| aae-mir-2b | 148         | 147                                         | 1               | 1.069 |
| aae-mir-12 | 145         | 132                                         | 13              | 1.2 |
| aae-mir-1891-2 | 120      | 41                                          | 79              | 3.1 |
| aae-mir-1891-1 | 120      | 41                                          | 79              | 3.1 |
| aae-mir-133 | 109         | 25                                          | 84              | 4.6 |
| aae-mir-252 | 105         | 202                                         | −97             | 0.6 |
| aae-mir-970 | 98          | 95                                          | 3               | 1.1 |
| aae-mir-306 | 88          | 79                                          | 9               | 1.2 |
| aae-mir-71 | 85          | 119                                         | −34             | 0.8 |
| aae-mir-279 | 46          | 131                                         | −85             | 0.4 |
| aae-mir-190 | 40          | 54                                          | −14             | 0.8 |
| aae-mir-305 | 35          | 55                                          | −20             | 0.7 |
| aae-mir-996 | 24          | 77                                          | −53             | 0.3 |
| aae-mir-210 | 18          | 808                                         | −790            | 0.0 |
| aae-mir-932 | 10          | 141                                         | −131            | 0.1 |
| aae-mir-285 | 1           | 114                                         | −113            | 0.0 |

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count of 2134 followed by aal-mir-13 and aal-mir-43a at 1874 and 1200 reads respectively (Table 4). Highly expressed miRNAs in CHIKV-infected *Ae.albopictus* saliva were aal-mir-43b, aal-mir-43a, aal-mir-413a, aal-mir-5 and aal-mir-249 with read counts of 4339, 2253, 1643, 1035 and 1032 respectively (Table 4).

**Relative abundance of miRNAs in CHIKV-infected saliva**

In comparison with uninfected *Ae.aegypti* saliva, CHIKV-infected saliva miRNA reads were slightly lower out of which 251277 reads corresponded with previously identified *Ae. aegypti* miRNAs (Table 1) and 54617 reads were novel miRNAs (Table 2). The highly expressed miRNAs, aae-mir-bantam, aae-mir-263a, aae-mir-125 and aae-mir-285 were upregulated in CHIKV-infected *Ae.aegypti* saliva with counts of 18002 (2.0-fold), 20584 (2.6-fold), 15735 (3.0-fold) and 10006 (>100-fold) when compared with uninfected read counts (Table 1). The novel miRNAs also did not demonstrate a significant total fold difference between the uninfected and infected saliva total read counts but individual miRNAs demonstrated differential expression (Table 2). In comparison with uninfected reads, highly expressed aae-mir-23, aae-mir-576 and aae-mir-320 were upregulated in CHIKV-infected *Ae.aegypti* saliva (Table 2) however aae-mir-80 was highly expressed in uninfected saliva (3773) but undetected in infected saliva.
Similar to *Ae.aegypti*, aae-mir-8 was also highly expressed at 12874 reads in uninfected *Ae. albopictus* saliva but in contrast to *Ae.aegypti*, was detectable in CHIKV-infected *Ae.albopictus* saliva (Table 3). Aae-mir-2940 was also downregulated (0.8-fold) in CHIKV-infected *Ae.albopictus* saliva whereas aae-mir-125 (2.2-fold), aae-mir-263a (3.4-fold), aae-mir-184 (1.3-fold) and aae-mir-100 (2.0-fold) were all upregulated in comparison with uninfected *Ae.albopictus* saliva. The highly expressed novel miRNAs, aal-mir-43b (1-fold), aal-mir-43a (0.9-fold), aal-mir-413a (>100-fold), aal-mir-5 (1-fold) and aal-mir-249 (0.7-fold) were all upregulated in CHIKV-infected *Ae.albopictus* saliva in comparison with uninfected *Ae.albopictus* saliva with the exception of aal-mir-413a, which was not detected in uninfected saliva at all (Table 4). MicroRNA aal-mir-13 was highly expressed in uninfected *Ae.albopictus* saliva but was undetected in CHIKV-infected saliva.

*Aedes* spp. saliva miRNAs modulate viral replication in mosquito and mammalian cells

In order to investigate the role of saliva miRNAs in the CHIKV replication, miRNA inhibitors were designed and transfected into mosquito (AAG-2 and C3/36) and mammalian (BHK-21) cells to profile CHIKV replication over time. In all three cell lines, there were no significant differences in CHIKV replication in non-transfected cells (CHIKV only), mock transfected cells (Transfected +CHIKV) and Scramble transfected cells. CHIKV replication in Scramble control cells peaked at 6.62 ± 0.03 log_{10} PFU/mL at 48 hours post infection (h.p.i.) in AAG-2 cells. In Scramble BHK-21 cells and C6/36 cells, CHIKV peaked at 48 h.p.i. with a titer of 7.41 ± 0.15 and 8.88 ± 0.21 log_{10} PFU/mL, respectively. **AAG-2 cells:** At 24-48 h.p.i., CHIKV titers were significantly lower (p < 0.05) in cells transfected with MIR-12 (Fig. 2A), MIR-125 (Fig. 2B) and mir-2490 (Fig. 2E) than in Scramble cells. At 48 h.p.i., CHIKV titers were significantly lower (p < 0.05) in cells transfected with MIR-184 (Fig. 2C) and MIR-375 (Fig. 2D). CHIKV titers peaked at 72 h.p.i. in AAG-2 cells transfected with miRNA inhibitors demonstrating an attenuated growth pattern compared to Scramble control cells where CHIKV titers peaked at 48 h.p.i. **BHK-21 cells:** Cells transfected with MIR-12 and MIR-125 did not exhibit any significant differences in CHIKV titers at any timepoint when compared with Scramble control cells. At 24 h.p.i., MIR-184 inhibited cells showed a significantly lower CHIKV titer of 7.16 ± 0.12 log_{10} PFU/mL in comparison to 7.5 ± 1.2 log_{10} PFU/mL (p < 0.05). CHIKV titers were significantly lower (p < 0.05) in MIR-375 and MIR-2940 inhibited cells at both 24 and 48 h.p.i. No significant viral titer differences were observed at 72 h.p.i. for any miRNA inhibitor. **C6/36 cells:** No significant differences were observed in titers for any miRNA inhibitor with the exception of MIR-184. At 24 and 48 h.p.i., CHIKV titers were 7.49 ± 0.29 and 8.40 ± 0.20 log_{10} PFU/mL respectively, which was significantly lower in comparison with Scramble control cells at those timepoints (p < 0.05).

**Discussion**

MicroRNAs are generally considered to be intra-cellular. However circulating microRNAs have also been identified from human serum, saliva and other biofluids [37–40] but have not been described before in mosquito saliva. In the present study, mature microRNAs were discovered in the saliva of two species of *Aedes* spp. mosquitoes, *Ae.aegypti* and *Ae.albopictus*. To our knowledge, this is the first documentation of the presence of exogenous miRNAs in mosquito saliva where at least 70% of these miRNAs were found within the *Ae.aegypti* and related *Anopheles gambiae* known miRNA databases. These miRNAs were mosquito-specific, insect-specific or were both insect and mammal specific. Notably, 30% of these discovered miRNAs were not found in the known miRNA database and were designated novel mosquito miRNAs.
Similar miRNAs were identified in both species of mosquitoes which corresponds with previous studies with *Ae.albopictus* and *Ae.aegypti* mosquito miRNAs [18] thus indicating the evolutionary pressure for miRNA sequence conservation and also potential multiple functions of each miRNA. Interestingly, the same miRNAs were highly expressed in both *Ae.albopictus* and *Ae.aegypti* saliva and these include aae-mir-8, aae-mir-2940, aae-mir-263a, aae-mir-bantam, aae-mir-125, aae-mir-184, aae-mir-281 and aae-mir-100 all of which have been identified in *Aedes* spp. before [18].

Recent studies have shown exosomes to be the extracellular vesicles that transport miRNAs in biofluids like saliva and serum [37,41,42]. Microvesicles, such as exosomes, play a major role in intercellular communication and has been shown to transfer functional and intact proteins, lipids and nucleic acids between cells. The argonaute family of proteins has also been shown to transport miRNAs via serum [43]. Studies with Epstein-Barr virus (EBV) have demonstrated infected B cells releasing exosomes that contain EBV-miRNAs [44]. Therefore it is possible that exosomes or argonaute proteins are transporting miRNAs from the mosquito salivary glands to the bite site via saliva to potentially modulate viral replication.

The miR-184 was highly expressed in both species. High expression of miR-184 has been reported in other insects as well [18,45] where miR-184 is ubiquitously expressed in varying levels at all stages of *Drosophila* development [31]. In comparison with uninfected saliva, aae-mir-184 was highly expressed but downregulated in CHIKV infected *Ae.aegypti* saliva and upregulated in infected *Ae.albopictus* saliva. In our miRNA inhibition assays, CHIKV replication was inhibited in AAG-2 and BHK-21 cells at 48 and 24 h.p.i but not at 72 h.p.i. This corresponds with a previous study, where upregulation of miR-184 was observed in *S. frugiperda* cells after baculovirus infection at 24 h.p.i. but downregulated by 72 h.p.i. and could potentially explain the lack of CHIKV inhibition in our study at 72 h.p.i. [31]. Significant inhibition of CHIKV replication in both AAG-2 and BHK-21 cells also indicates the important role of miR-184 in arboviral infections in both mosquito and mammalian host. MicroRNA-184 has also been shown to increase in response to interleukin-22 (IL-22), a proinflammatory cytokine associated with inflammatory skin disorders, thereby reducing expression of Argonaute-2 (AGO 2) protein in human keratinocytes [46]. The AGO 2 protein recognizes and cleaves targeted dsRNA as part of the RNA-induced silencing complex (RISC) in the RNA interference (RNAi) pathway. As the RNAi pathway is an important defense pathway against viral infections in several mosquito species [47–50] differential expression of aae-miR-184 post-infection in mosquitoes could modulate AGO 2 levels thereby regulating viral replication at the initial site of infection. The C6/36 cell line has a dysfunctional RNAi pathway where Dicer-2, part of the RISC that associates with AGO 2, is lacking [51,52]. In the present study, CHIKV replication was inhibited at 24 and 48 h.p.i. in C6/36 cells suggesting a potentially more complex role of miR-184 in the RISC.

The highly expressed aae-miRNA-125 and aae-miR-100 were both upregulated in CHIKV-infected *Ae.aegypti* and *Ae.albopictus* saliva. MicroRNA-125, a homolog of *Drosophila* miR-let-7, is expressed in specific developmental stages of Drosophila [53]. MicroRNA-125, miR-100 and miR-let-7 are part of the same primary transcript and originate from a common genomic locus in *Drosophila* [54]. Additionally, clustering of the paralogs of these miRNAs also exists in the mouse genome suggesting multiple roles of these miRNAs across different species [55,56]. Target sites for mir-125a and mir-125b have been predicted to be within the 3’UTR of both...
mouse and human TNF-α transcripts [57] and miR-125b levels either increase or decrease in response to TNF-α stimulated macrophages both in vitro and in vivo [57]. Additionally, down-regulation of TNFAIP results in increased levels of NF-κB which contributes to increased immune cell activity [58]. Therefore, both aae-mir-125 and aae-mir-100 could be contributing to regulating immune cell activity at the bite site in order to influence CHIKV replication.

The aae-miR-375 has been shown to be important in DENV replication [21] and was down-regulated at least 34-fold in Ae.aegypti and undetected in Ae.albopictus in the present study. Predicted target sites for miR-375 include the REL1 and prohibitin, the 5'UTR of cactus, the 3'UTR of DEAD box ATP-dependent RNA helicase, a hypothetical protein and the coding region of kinesin all of which showed significant modulation in response to Ae.aegypti mosquitoes injected with aae-miR-375 mimics [21]. Cactus and REL1 regulate the Toll immune pathway and were differentially expressed in response to aae-miR-375 mimics in Ae.aegypti mosquitoes and AAG-2 cells [21]. Furthermore, presence of aae-miR-375 mimics increased DENV-2 levels in AAG-2 cells which corresponded with our miRNA inhibition assay where a decrease in CHIKV replication was observed in AAG-2 and BHK-21 cells after exposure to aae-miR-375 inhibitors. As the cactus gene inhibits NF-κB transcription factor activation, it seems that aae-miR-375 allows for enhanced virus infection in AAG-2 cells via downregulation of cactus. Indeed, DENV infection was attenuated when the cactus gene was silenced Ae.aegypti mosquitoes [59]. In another study, miR-375 function was enhanced by increased expression of AGO2 in mice suggesting a potential interaction of aae-miR-375 and AGO2 [60].

In AAG-2 cells and BHK-21 cells, aae-miR-2490 inhibitors significantly reduced CHIKV replication at 24 and 48 h.p.i. which corresponds with reduced Wolbachia replication in AAG-2 cells exposed to aae-miR-2490 inhibitors [25]. Additionally, the aae-miR-2940 has been shown to target and upregulate metalloprotease m41 fsh expression in AAG-2 cells and Ae. aegypti mosquitoes after Wolbachia infection which enhances its replication [25]. In another study, aae-miR-2490-5p was shown to enhance West Nile virus replication in C6/36 cells but not aae-miR-2490-3p. In contrast, in our study, CHIKV replication was unaffected by aae-miR-2490-3p inhibition in C6/36 cells as the aae-miR-2490 inhibitor was designed against aae-miR-2490-3p due to the predominant number of read counts in the saliva (aae-miR-2490).

The aae-miR-12 was highly upregulated in CHIKV-infected Ae.aegypti saliva but was unaffected in Ae.albopictus saliva. In cells transfected with aae-miR-12 inhibitors, reduced CHIKV replication was observed in AAG-2 cells but not BHK-21 or C6/36 cells. While aae-miR-12 has not been characterized with viruses, a similar pattern was observed in AAG-2 cells inhibition of aae-miR-12 greatly reduced Wolbachia density [25]. Potential targets of aae-miR-12 were predicted to be MCM6(DNA replication licensing factor), MCT1 (monocarboxylate transporter) and the Exonuclease gene however only MCM6 and MCT1 were down-regulated when exposed to aae-miR-12 mimics in AAG-2 cells [24].

Out of the 5 miRNAs inhibited, all demonstrated lower CHIKV titers in AAG-2 cells however, only miR-184, miR-375 and miR-2490, demonstrated decreased CHIKV titers in both mosquito (AAG-2) and mammalian (BHK-21) cells. This suggests a multiple roles and multiple target sites of these miRNAs across various species. It further suggests that these 5 miRNAs, along with the other highly expressed discovered miRNAs, could be acting in concert at the bite site to regulate viral replication, viral dissemination and immune cell activity in the host. Because inhibiting the miRNAs decreased viral replication, the presence of these miRNAs and upregulation of these miRNAs in mosquito saliva most likely enhances CHIKV replication and dissemination in the host and at the site of infection.

In conclusion, we have discovered microRNAs from mosquito saliva and have identified saliva miRNAs that are expressed only upon CHIKV infection. To our knowledge, this is the first report on the identification of exogenous mosquito saliva microRNAs. Identification of several
miRNAs only in the CHIKV infected saliva suggests a possible importance in CHIKV transmission and establishment of infection in the host. Though the functional roles of these miRNAs are yet to be established, our in-vitro data from testing 5 miRNAs demonstrate their role in the regulation of CHIKV infection. These miRNAs may play an important role in regulating the establishment of CHIKV infection in the mammalian host during blood feeding, and are a subject of our future study.

Supporting Information

S1 Fig. Predicted stem-loop structures of novel microRNAs. The miRDeep2 software was used to confirm the presence of miRNAs based on nucleotide length, star sequence, stem-loop folding and homology to AaegL1 and AgamP3 genomes. Figures a) aae-miR-249 b) aae-miR-23 c) aal-miR-43b and d) aal-miR-5 show the predicted stem-loop structures, star and mature sequences of highly expressed novel microRNAs in Ae.aegypti and Ae.albopictus saliva. As the Ae.albopictus genome has not been described, aal-miR, was used to designate novel miRNAs identified in Ae.albopictus. (TIFF)

Author Contributions

Performed the experiments: PDM JH SGW ST. Analyzed the data: PDM SGW TGW ST. Contributed reagents/materials/analysis tools: PDM SGW TGW ST. Wrote the paper: PDM SGW ST. Conceived the idea: ST. Designed experiments: ST PDM.

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