

RESEARCH ARTICLE

# 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

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](http://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% CO<sub>2</sub>. 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 CO<sub>2</sub>. 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 CO<sub>2</sub>. The infectious clone, CHIKV-LR 5' GFP (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 5' GFP 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 5' GFP: 4.6 TCID<sub>50</sub>/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  $-20^{\circ}\text{C}$ . After 24 hours post-inactivation, RNA samples were thawed and  $150\mu\text{L}$  of chloroform was added to each tube and shaken vigorously for 30 seconds. The samples were centrifuged for 15 minutes at  $10000 \times g$  at  $4^{\circ}\text{C}$  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  $700\text{--}850 \text{ K/mm}^2$ . 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* (AeagL1) 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 (AeagL2 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<sup>®</sup>. 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 $\mu$ L were collected from each replicate until 72 hours post-infection, added to 450 $\mu$ L of diluent and stored at  $-80^{\circ}\text{C}$  until further processing. A standard plaque assay on Vero cells was used to determine CHIKV titer at each timepoint as previously described [36].

## Statistical analysis

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

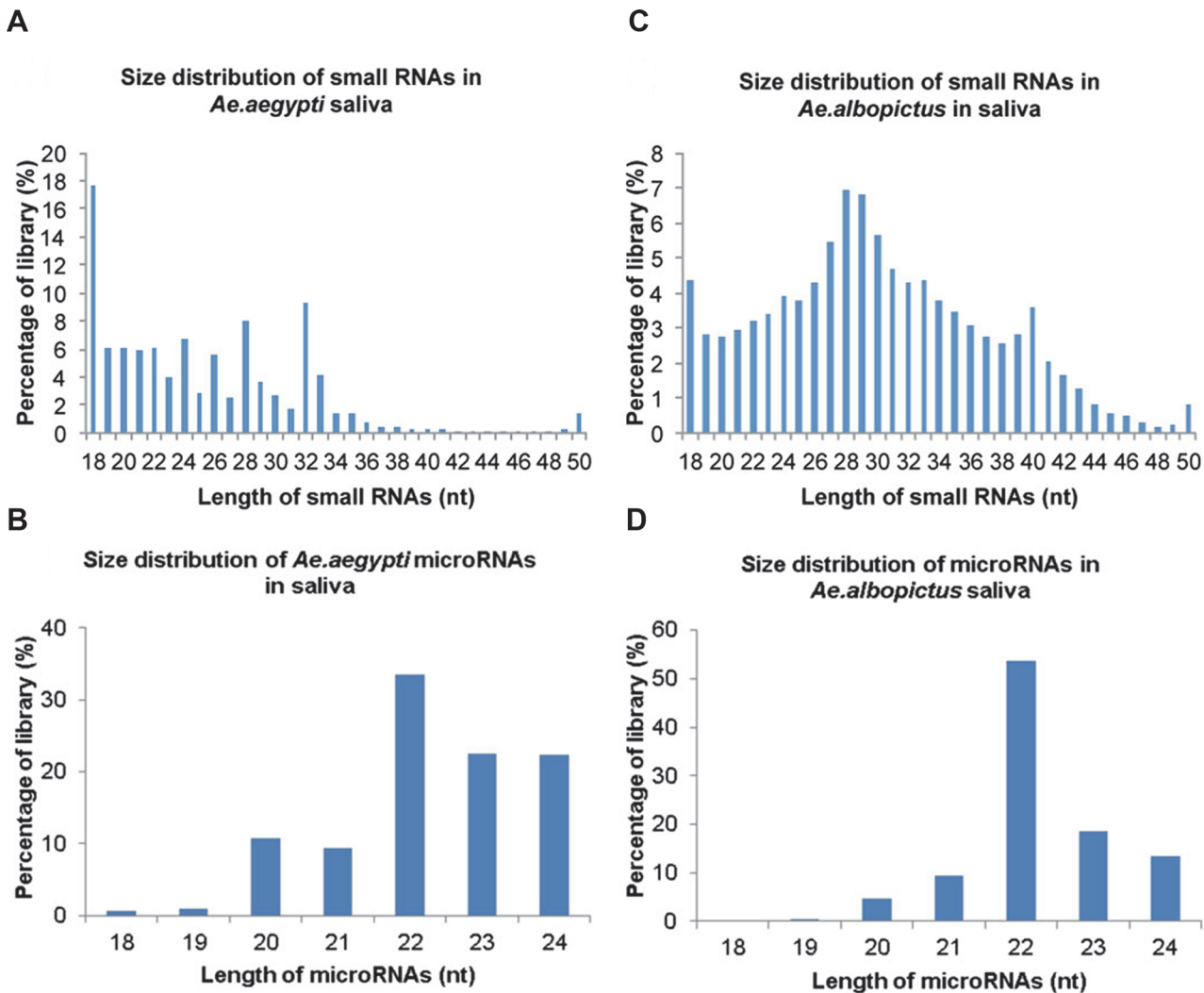
## Results

### 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  $14 \times 10^6$  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  $3 \times 10^6$  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-ae-249, miR-ae-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





**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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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),

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

MicroRNA	Read counts		Difference between infected and uninfected	
	Infected	Uninfected	Difference	Fold 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)

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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aae-mir-8 (47613), aae-mir-184 (10105) aae-mir-bantam (9969), aae-mir-263a (9084) and aae-mir125 (5863) (Table 1). 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).

### 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



**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
aee-mir-143	aaccgguagauccgaacuugug	13129	4275	8854	0.8
aee-mir-249	ucagucuuuuucucucuccu	12951	5385	7566	0.7
aee-mir-5	uaggaacuucuuaccgugcucu	9219	2566	6653	1.0
aee-mir-229	ucauaagacacacgcgcuau	544	259	285	0.6
aee-mir-778	uuggucccuucaaccagcugu	278	11	267	7.0
aee-mir-620	auuagaauugugaucuguuuu	51	3	48	4.7
aee-mir-3069	uuuguucguuuggcucgagu	54	188	-134	0.1
aee-mir744	caucacagucugaguucucugcu	1451	1783	-332	0.2
aee-mir-115	ugugaugugacguagugguac	71	616	-545	0.0
aee-mir-23	uagcaccuuucgaaucagucac	10021	0	10021	10021.0
aee-mir-576	ggggauaguagcucagugguagag	2033	0	2033	2033.0
aee-mir-320	uuucggauauguuuagaaauuc	1262	0	1262	1262.0
aee-mir-214	uucccgagcagccccca	606	433	173	0.4
aee-mir-402	uucccgagcagccccca	606	433	173	0.4
aee-mir-65	ugcacacgacucgauugggagagac	397	0	397	397.0
aee-mir-341	gcaggauucgagggagcuc	294	0	294	294.0
aee-mir-3	agggucggagguucgaaucucc	250	0	250	250.0
aee-mir-3798	auauuguccugucacagcag	226	0	226	226.0
aee-mir-187	auauuguccugucacagcag	226	0	226	160.0
aee-mir-242	caucgaucgagcaccuga	160	0	160	139.0
aee-mir-309	guaggccgagcgaacuucuc	139	21	118	1.8
aee-mir-210	uuuaccuuuucagagacc	129	0	129	129.0
aee-mir-1571	gagagccuguguaauuc	88	0	88	88.0
aee-mir-1247	guucgacuccagucggu	81	12	69	1.9
aee-mir-40	uugcguugauuaaguccc	81	0	81	81.0
aee-mir-843a	guccugucagcggucgcca	74	0	74	81.0
aee-mir-117	uagcagaauccugaguaggac	73	0	73	74.0
aee-mir-360	gugagcauuuuucagggugugu	67	0	67	73.0
aee-mir-359	guaacugacgucgaggag	56	0	56	67.0
aee-mir-80	auucucugucgucaccca	0	3773	-3773	0.0
aee-mir-109	aucacgucgggucaccca	0	227	-227	0.0

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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 aee-mir-8 with a count of 12874 followed by aee-mir-2940 (2574), aee-mir-bantam (2127) and aee-mir-125 (2132) (Table 3). Highest read counts in CHIKV-infected *Ae.albopictus* saliva were from aee-mir-125 (4333), aee-mir-263a (4293), aee-mir-8 (2609), aee-mir-184 (2332) and aee-mir-100 (2255) (Table 3). With the exception of aee-mir-8, these miRNAs were up-regulated 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

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

MicroRNAs	Read Counts		Difference between Infected and Uninfected	
	Infected	Uninfected	Difference	Fold 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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**Table 4.** Read counts of individual novel microRNAs detected in uninfected and CHIKV-infected *Ae.albopictus* saliva.

Assigned Name	Consensus sequence	Read Counts		Difference between infected and uninfected	
		Infected	Uninfected	Difference	Fold Difference
aal-mir-43a	aacccguagauccgaacuugug	2253	1200	1053	0.88
aal-mir-5	uaggaacuucacaccgugcucu	1035	482	553	1.00
aal-mir-249	ucagucuuuuucucucuccu	1032	729	303	0.66
aal-mir-778	uuggucccucaaccagcugu	108	25	83	2.02
aal-mir-774	caucacagucugaguucuuugcu	378	336	42	0.53
aal-mir-229	ucauaagacacacgcgcuau	98	95	3	0.48
aal-mir-43b	ucccgagaccuaacuuguga	4339	2134	2205	0.95
aal-mir-413a	guucgaauccugucugg	1643	0	1643	1643.00
aal-mir-305	guucgaauccugucgag	984	0	984	984.00
aal-mir-6	guucgaauccuguaaga	892	0	892	892.00
aal-mir-47	ggggauguagcucagugguagag	751	0	751	751.00
aal-mir-157	uguggcguaguugguac	398	0	398	398.00
aal-mir-28	guggagcaguauuggaagc	373	0	373	373.00
aal-mir-69	guggcguaauugguagac	267	0	267	267.00
aal-mir-137	aggucgugguucgaacccc	232	0	232	232.00
aal-mir-2308	ggucggugucgaaucc	119	0	119	119.00
aal-mir-214	uucccgagcagcccca	117	201	-84	0.27
aal-mir-309	guaggccgagaaacuugc	82	36	46	1.07
aal-mir-62	acgucuuuucauuguc	80	103	-23	0.36
aal-mir-143	acccgugaaggaguuucggag	71	0	71	71.00
aal-mir-127	guagccagaggaagagaaa	61	0	61	61.00
aal-mir-446	ucuuuuuuuuucgucgccc	53	0	53	53.00
aal-mir-408	guucgaauccuagucggga	52	0	52	52.00
aal-mir-13	uggacggagaacugauagggc	0	1874	-1874	0.00

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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.

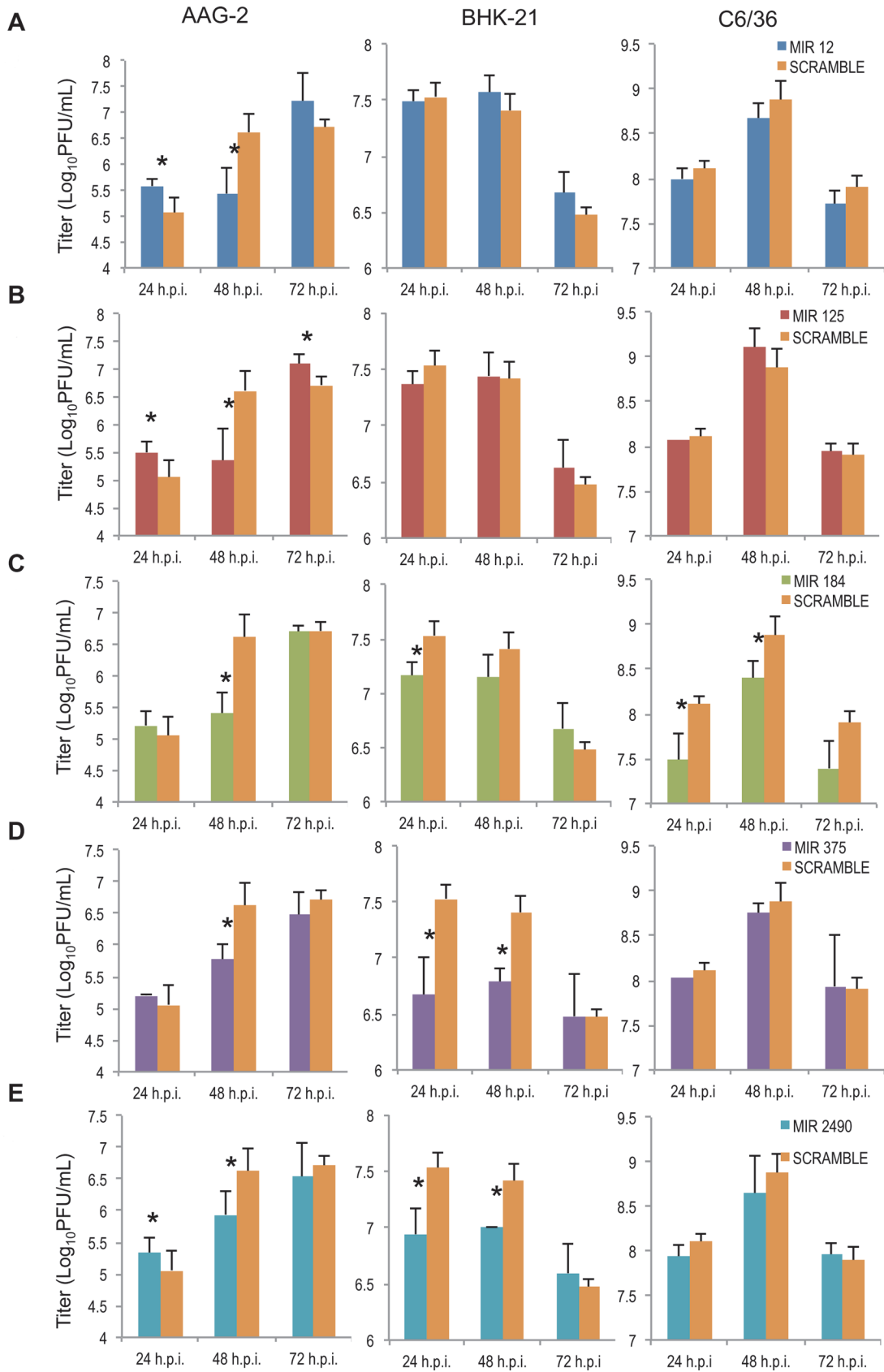
Similar to *Ae.aegypti*, aae-mir-8 was also highly expressed at 12874 reads in uninfected *Ae. albopictus* saliva but in contrast to *Ae.aegypti*, was detectable in CHIKV-infected *Ae.albopictus* saliva (Table 3). Aae-mir-2940 was also downregulated (0.8-fold) in CHIKV-infected *Ae.albopictus* saliva 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 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 C6/36) 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  $6.62 \pm 0.03 \log_{10}$ 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  $7.41 \pm 0.15$  and  $8.88 \pm 0.21 \log_{10}$ 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  $7.16 \pm 0.12 \log_{10}$ PFU/mL in comparison to  $7.5 \pm 1.2 \log_{10}$ 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  $7.49 \pm 0.29$  and  $8.40 \pm 0.20 \log_{10}$ 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.



**Figure 2. Saliva microRNAs regulate CHIKV replication in mosquito and mammalian cells.** Mosquito (AAG-2 and C6/36) and mammalian (BHK-21) cells were transfected with miRNA inhibitors, a) MIR-12, b) MIR-125, c) MIR-184, d) MIR-375 and e) MIR-2940, and then infected with CHIKV at 72 hours post-transfection. Supernatant was collected daily for 72 hours and viral titers for each timepoint were determined via standard plaque assay on Vero cells.

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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 nuclei 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- $\alpha$  transcripts [57] and miR-125b levels either increase or decrease in response to TNF- $\alpha$  stimulated macrophages both *in vitro* and *in vivo* [57]. Additionally, down-regulation of TNFAIP results in increased levels of NF- $\kappa$ B 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- $\kappa$ B 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-2490 has been shown to target and upregulate metalloprotease m41 fth 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 *Ae.g1* 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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