AFRL-SA-WP-TR-2019-0009

Therapeutic Human miRNAs for Use as Anti-Viral Drugs for Influenza A Infection

Samina N. Assanie-Shivji (Samina N. Hassanali), PhD
James Baldwin, PhD

March 2019

Final Report
for September 2015 to September 2016

Air Force Research Laboratory
711th Human Performance Wing
U.S. Air Force School of Aerospace Medicine
Aeromedical Research Department
2510 Fifth St., Bldg. 840
Wright-Patterson AFB, OH 45433-7913

NOTICE AND SIGNATURE PAGE

Using Government drawings, specifications, or other data included in this document for any purpose other than Government procurement does not in any way obligate the U.S. Government. The fact that the Government formulated or supplied the drawings, specifications, or other data does not license the holder or any other person or corporation or convey any rights or permission to manufacture, use, or sell any patented invention that may relate to them.

Qualified requestors may obtain copies of this report from the Defense Technical Information Center (DTIC) (http://www.dtic.mil).

AFRL-SA-WP-TR-2019-0009 HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION IN ACCORDANCE WITH ASSIGNED DISTRIBUTION STATEMENT.

//SIGNATURE//	//SIGNATURE//
COL ALBERT H. BONNEMA	DR. RICHARD A. HERSACK
Chief, Applied Technology & Genomics Div	Chair, Aeromedical Research Department

This report is published in the interest of scientific and technical information exchange, and its publication does not constitute the Government's approval or disapproval of its ideas or findings.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From – To)*
28 Mar 2019	Final Technical Report	September 2015 – September 2016

4. TITLE AND SUBTITLE

Therapeutic Human miRNAs for Use as Antiviral Drugs for Influenza A Infection

5a. CONTRACT NUMBER
FA8650-12-D6280

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Samina N. Assanie-Shivji (Samina N. Hassanali), James Baldwin

5d. PROJECT NUMBER

5e. TASK NUMBER
0034

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Prime Contractor:
Wyle Laboratories, Inc.
1290 Hercules
Houston, TX 77058

Subcontractor:
Claflin University
400 Magnolia St.
Orangeburg, SC 29115

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

USAF School of Aerospace Medicine
Aeromedical Research Dept
Applied Technology & Genomics Div
2510 5th St., Bldg. 840
Wright-Patterson AFB, OH 45433-7913

10. SPONSORING/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
AFRL-SA-WP-TR-2019-0009

12. DISTRIBUTION / AVAILABILITY STATEMENT

DISTRIBUTION STATEMENT A. Approved for public release. Distribution is unlimited.

13. SUPPLEMENTARY NOTES

This is a QUASAR FINAL REPORT under Contract FA8650-12-D-6280, Task Order 0034. Report contains color. Cleared, 88PA, Case # 2019-2063, 29 Apr 2019.

14. ABSTRACT

This report describes activity of 5 common up-regulated and 5 common down-regulated microRNAs, polyarginine-tagged on influenza A H1N1 and H3N2 subtype replication.

15. SUBJECT TERMS

Influenza A, H1N1, H3N2, nasal wash samples

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Dr. James Baldwin
U	U	U	SAR	23	19b. TELEPHONE NUMBER *(include area code)*

This page intentionally left blank.

TABLE OF CONTENTS

Page

LIST OF FIGURES ii
LIST OF TABLES ii
1.0 SUMMARY 1
2.0 INTRODUCTION 1
3.0 METHODS, ASSUMPTIONS, AND PROCEDURES 2
3.1 Nasal Wash Acquisition 2
3.2 RNA Extraction 2
3.3 qRT-PCR Analysis to Detect H1N1 and H3N2 2
3.4 Gel Electrophoresis 4
3.5 miRNA Profiling 4
3.6 Data Analysis 5
3.7 Polyarginine Peptide and Cy3 Tagging to miRNAs 5
3.8 MDCK Transfection with H1N1 and H3N2 and Treatment with 10 Potential Identified miRNAs 5
3.9 H1N1 and H3N2 Localization by Immunostaining in Transfected and Untransfected MDCK Cells Treated with and without 10 Potential Identified miRNAs 5
3.10 Detection of H1N1 and H3N2 Load in Transfected, Untransfected, and Treated MDCK Cells by qRT- PCR 6
4.0 RESULTS AND DISCUSSION 6
4.1 Selection of 50 Nasal Washes 6
4.2 Confirmation of Negative Controls, and H1N1 and H3N2 Positive Nasal Wash Samples by Gel Electrophoresis 8
4.3 miRNA Profiling and Analysis 10
4.4 Up-Regulated miRNAs Reduces Cytopathic Effect in H1N1 and H3N2 Transfected and Treated MDCK Cells 11
4.5 Up-Regulated miRNAs Reduces H1N1 and H3N2 Infection in Treated MDCK Cells 12
4.6 Up-Regulated miRNAs Decreases H1N1 and H3N2 Load 14
5.0 CONCLUSIONS 16
6.0 REFERENCES 16
LIST OF ABBREVIATIONS AND ACRONYMS 17

LIST OF FIGURES

Page

Figure 1. Gel Electrophoresis of Selected Nasal Wash Samples Positive for H1N1 and H3N2 9
Figure 2. Gel Electrophoresis of Selected Nasal Wash Samples Positive for H3N2 and Negative for H1N1 and H3N2 10
Figure 3. Increase in H1N1 and H3N2 localization in Untreated MDCK cells 12
Figure 4. Increase in H1N1 and H3N2 localization in MDCK cells Treated with hsa-miR-1233-5p 13
Figure 5. Decrease in H1N1 and H3N2 localization in MDCK cells Treated with hsa-miR-3154..... 14
Figure 6. Effect of 5 common down-regulated miRNAs and 5 common up-regulated miRNAs on H1N1 and H3N2 load over 5 weeks in MDCK Cells by qRT-PCR..... 15

LIST OF TABLES

Page

Table 1. Primers Used for Detection of H1N1 and H3N2 in Nasal Wash Samples (In-House) 3
Table 2. Primers and Probes Used for Detection of H1N1 and H3N2 in Nasal Wash Samples (DoD) 3
Table 3. qRT-PCR Reaction Mix Contents 3
Table 4. qRT-PCR Cycling for In-House Primers and DoD Primers and Probes 4
Table 5. Twenty Nasal Wash Samples Positive for H1N1 Using In-House Primers 7
Table 6. Twenty Nasal Wash Samples Positive for H3N2 Using In-House Primers 7
Table 7. Ten Nasal Wash Samples Negative for H1N1 and H3N2, and Two Positive Controls: Isolate H1N1 and Isolate H3N2 Positive for H1N1 and H3N2, Using In-House Primers 8
Table 8. Common MicroRNAs Expressed in H1N1 and H3N2 Infected Nasal Wash Samples .. 11

1.0 SUMMARY

The aims of this research project were to: 1) analyze the differential expression of microRNAs (miRNA) in 50 nasal wash samples in response to influenza A (subtype: H1N1 and H3N2) infections; 2) identify 5 common up-regulated and 5 common down-regulated expressed miRNAs; and, 3) determine the inhibitory effect of the 5 common up-regulated and 5 common down-regulated expressed polyarginine-tagged miRNAs on influenza A H1N1 and H3N2 subtype replication.

In this study, we found that miRNAs that were down-regulated in microarray profile increased the influenza A H1N1 and H3N2 subtype replication. Whereas, the miRNAs that were up-regulated in microarray profile decreased the influenza A H1N1 and H3N2 subtype replication. Human miRNA-1233-5p that was down-regulated -2.76 fold and -25.64 fold for H1N1 and H3N2, respectively, increased the H1N1 and H3N2 load. Whereas, Human miRNA-3154 that was up-regulated 11.11 fold and 12.33 fold for H1N1 and H3N2, respectively lowered the H1N1 and H3N2 load.

2.0 INTRODUCTION

Influenza is a contagious disease that causes respiratory tract infections that accounts for several hospitalizations and deaths per year. The “flu” is caused by the influenza virus and is spread by bioaerosols through coughing, sneezing and close contact with an infected person. The virus spreads seasonally, with its peak in the winter months, beginning in October and continuing through May. There are three strains of influenza viruses: A, B and C, and are further characterized by the glycoproteins hemagglutinin (HA), which aids in binding to the target and viral entry, and neuraminidase (NA), which releases the virus in the cell [1]. Both HA and NA are present on the outer surface of the virus [2]. The number associated with the H an N in the strain name identifies how many of each protein is on the virus particle [1].

The influenza virus is a single-stranded, negative-sense RNA virus, spherical in shape, surrounded by a lipid envelope, and encapsulated [3]. The virus is about 80-120 nanometers (nm) in length and genome is about 13,588 bases long [3, 4].

Each year, approximately 500,000 deaths are caused due to flu related illnesses. Recurrently, new strains emerge over time, for example, the H1N1 virus in 2009, causing significant morbidity and mortality that continues to be a matter today [2]. This caused a huge expense for our military, and the main cost of this was due to the need of additional vaccination and health care for the children who were getting infected that were less than 5 years of age [5].

The flu is not only seen in the United States, but also high percentages have been reported in other countries. Several countries, including Brazil and Eastern Europe, show high percentages of respiratory specimens that tested positive for influenza, were mostly subtype A [6]. United Kingdom is still reporting high rates of influenza-related hospitalization, having percentages greater than 30% 6]. Although cases of Influenza B are being seen globally, but subtype A is prevalent amongst several countries globally [6].

The main objectives of this project were to analyze the differential expression of microRNAs (miRNA) in response to influenza A (subtype: H1N1 and H3N2) infection of 50 nasal was samples, using microarray profiling, and identifying new potentially important diagnostic and therapeutic human miRNA targets for inhibiting influenza A replication. We

identified five common up-regulated and five common down-regulated miRNAs through microarray analysis. These miRNAs were tagged with polyarginine and Cy3, and used for treating H1N1 and H3N2 Madin-Darby Canine Kidney (MDCK) infected cells. Our results show that human miRNA (has-miR) hsa-miR-1233-5p that was down-regulated -2.76 fold and -25.64 fold for H1N1 and H3N2, respectively, increased the H1N1 and H3N2 load. Whereas, hsa-miR-3154 that was up-regulated 11.11 fold and 12.33 fold for H1N1 and H3N2, respectively lowered the H1N1 and H3N2 load. Our findings also show that all up-regulated miRNAs: hsa-miR-3154, hsa-miR-3173-3p, hsa-let-7e-5p, hsa-miR-3691-5p and hsa-miR-101-5p, decreased the viral load and viral localization in infected MDCK cells, but hsa-miR-3154 was more pronounced in inhibiting both H1N1 and H3N2 load and localization in infected MDCK cells. Hence, our in vitro findings show that the up-regulated miRNAs can be used as an alternative approach for treating influenza A infection based on miRNA-mediated gene silencing.

3.0 METHODS, ASSUMPTIONS, AND PROCEDURES

3.1 Nasal Wash Acquisition

Dr. Assanie-Shivji obtained 128 nasal wash samples from Dr. James Baldwin at the DoD Applied Technology and Genomics Division, WPAFB, OH coordinated with Sgt. Charlee A. Martin. Of these 128 nasal wash samples, 52 nasal wash samples from individuals of ages 18-65 years were used in this study. These 52 nasal wash samples included two positive controls: H1N1 isolate and H3N2 isolate, 20 positive influenza A H1N1: 10 male and 10 female, 20 influenza A H3N2: 10 male and 10 female and 10 negative influenza A control: five male and five female.

3.2 RNA Extraction

The total RNA was extracted from 128 nasal wash samples using the miRNeasy Serum/Plasmid Kit (Cat # 217184, Qiagen GmbH, Hilden, Germany) according to manufacturer's instructions. Purified RNA quantitation was determined by measuring the optical density at 260/280 nm using NanoDrop Lite (Thermo Scientific, Wilmington, DE). The purified RNA was stored at −80°C until use.

3.3 qRT-PCR Analysis to Detect H1N1 and H3N2

Influenza A is an RNA virus, so first cDNA synthesis was done which was included in quantitative reverse transcription polymerase chain reaction (qRT-PCR) cycling, using iTaq™ Universal SYBR® Green One-Step Kit (Cat # 172-5151, BIO-RAD, Hercules, CA) according to manufacturer's instructions. Detection of H1N1 and H3N2 was performed using in-house primers (Table 1), and DoD primers and probes (Table 2) provided by Dr. Baldwin. Contents of the tubes and qRT-PCR cycling are shown in Table 3 and Table 4, respectively. Each qRT-PCR run included a negative control having water and no RNA. qRT-PCR was performed in CFX Connect Real-Time System (BIO-RAD, Hercules, CA).

Table 1. Primers Used for Detection of H1N1 and H3N2 in Nasal Wash Samples (In-House)

Influenza A Subtype	Primer	Sequence
H1N1 NA	Forward	5′-GCGACCCAAAGAGAACACAA-3′
	Reverse	5′-CAACTCAGCACCGTCTGG-3′
H1N1 HA	Forward	5′-AGGATTGAGGAATGTCCCGTCTAT-3′
	Reverse	5′-ATCTACCATCCCTGTCCACCC-3′3’
H3N2 NA	Forward	5′-GGGATGGACAGGGATGGT-3′
	Reverse	5′-TCGTCAATGGCATTCTGTGT-3′
H3N2 HA	Forward	5′-GGAGGGAATGATAGACGGTTGGTA-3′
	Reverse	5′-CGATGGCTGCTTGAGTGCTT-3′

Table 2. Primers and Probes Used for Detection of H1N1 and H3N2 in Nasal Wash Samples (DoD)

Influenza A Subtype	Primer Probe	Sequence
PAN Influenza A	Forward	5′-GACCRATCCTGTCACCTCTGAC-3′
	Reverse	5′-AGGGCATTYTGGACAAAKCGTCTA-3′
	Probe	5′-/56-FAM/TGCAGTCCTCGCTCACTGGGCACG /3BHQ_1/-3′
H3N2	Forward	5′-AAGCATTCCYAATGACAAACC-3′
	Reverse	5′-ATTGCRAATATGCCTCTAGT-3′
	Probe	5′-/56-FAM/CAGGATCACATATGGGSCCTGTCCCAG/3BHQ_1/-3′

Table 3. qRT-PCR Reaction Mix Contents

In-House Primers		DoD Primers and Probes	
Reaction Mix	**Amount (µL)**	**Reaction Mix**	**Amount (µL)**
1X iTaq Universal SYBR Green Reaction mix	12.5	1X iTaq Universal SYBR Green Reaction mix	12.5
iScript Reverse Transcriptase	0.5	iScript Reverse Transcriptase	0.5
200nM Forward primer	0.5	40 µM Forward primer	0.5
200nM Reverse primer	0.5	40 µM Reverse primer	0.5
-	-	10 µM probe	0.5
Nuclease free water	10.0	Nuclease free water	9.5
RNA (5.0 ng)	1.0	RNA (5.0 ng)	1.0
Total	25.0	Total	25.0

Table 4. qRT-PCR Cycling for In-House Primers and DoD Primers and Probes

In-house Primers and Probes		DoD Primers and Probes	
Temperature (°C)	**Time**	**Temperature (°C)**	**Time**
50	10 min	50	30 min
95	1 min	95	2 min
95	10 sec[1]	95	15 sec[2]
60	10 sec[1]	55	30 sec[2]
72	10 sec[1]	72	30 sec[2]
72	5 min	-	-
65- 95	10 sec hold of 0.5 °C increase	-	-

[1]40 cycles
[2]45 cycles

3.4 Gel Electrophoresis

Amplified products of all 128 samples including two positive controls H1N1 and H3N2, and negative controls were separated by 2% agarose gel electrophoresis and viewed under the UV transilluminator.

3.5 miRNA Profiling

Qiagen Genomic Services (Qiagen, Fredrick, MD) profiled the human miRNAs (hsa-miR) for the 52 samples (50 nasal wash samples and H1N1 and H3N2 positive controls) using miScript miRNA PCR Array (Human miRNome, miRBase V21). Reverse transcription was conducted using miScript II RT Kit (Cat # 218161, Qiagen GmbH, Hilden, Germany). To each respective tube, 2 µL of 5x miScript HiSpec Buffer, 1 µL of 10x miScript Nucleics Mix, and 1 µL of miScript reverse transcriptase mix, along with the RNA and nuclease free water was added. Samples were then incubated for 60 min at 37 °C, then at 95 °C for 5 min to inactivate miScript reverse transcriptase mix. The next step was preamplification using miScript PreAMP PCR Kit (Cat # 331452, Qiagen GmbH, Hilden, Germany). The tubes were prepared respectively by adding 5 µL of 5x miScript PreAMP buffer, 2 µL of HotStar Taq DNA polymerase, 5 µL of miScript PreAMP primer mix, 7 µL of RNase-free water, 1 µL of miScript PreAMP universal primer and 5 µL of diluted template cDNA of 52 samples. Next, cycling was performed in a thermal cycler for preamplification using the program of 95 °C for 15 min, and then 12 cycles of 94 °C for 30 sec and 60 °C for 3 min. Once preamplification was completed, the preamplified cDNA was diluted in RNase-free water and stored on ice. Preamplified cDNA was used for real-time miScript miRNA PCR Array (Human miRNome, miRBase V21) in combination with miScript SYBR Green PCR Kit (Cat # 218076, Qiagen GmbH, Hilden, Germany) using ABI 7900 HT real-time PCR instrument.

3.6 Data Analysis

Ct values were obtained for 52 samples and normalized using the global Ct value for miRNA (Ct < 30). The data was analyzed by Qiagen using a gene globe that generated a fold expression and p-value for all off the 52 samples. A composite ranking for the miRNA was generated using the fold expression and the predicted p-value by Dr. Baldwin. The composite ranking was used to identify the potential 10 miRNA targets in inhibiting influenza A virus replication in MDCK cells.

3.7 Polyarginine Peptide and Cy3 Tagging to miRNAs

Dr. Assanie-Shivji sent the mature sequences of the 5 common miRNAs that were up-regulated and downregulated to Sigma-Aldrich for synthesis of miRNAs and tagging the miRNAs with polyarginine peptide and a fluorochrome (Cy3) at the 5' end of the mature miRNA sequence.

3.8 MDCK Transfection with H1N1 and H3N2 and Treatment with 10 Potential Identified miRNAs

MDCK cell line (ATCC® CCL34™) were first transfected with H1N1 RNA (ATCC VR-1736D™ and H3N2 RNA (ATCC VR-1680D™) with Lipofectamine® MessengerMAX™ Transfection Reagent (Cat # LMRN008, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After overnight transfection the serum free Eagle's Minimum Essential Medium (EMEM) (ATCC 302003™) was removed and pre-warmed complete EMEM having 10% fetal bovine serum and antibiotics was added and cells were incubated at 37 °C in 5% CO2. MDCK cells not infected with H1N1 and H3N2 served as a control. Both transfected and un-transfected MDCK cells were monitored daily for cytopathic effect (CPE) development for 10 days, until 80-90% confluency was obtained.

After 10 days of transfection on Day 11, transfected and un-transfected cells were treated with 1 ug of Dose 1 (Day 0: Dose 1 treatment) of the 10 potential identified miRNAs tagged with polyarginine peptide and Cy3 (5 common up-regulated and 5 common down-regulated) with Lipofectamine RNAiMAX Transfection Reagent (Cat # 13778075, Invitrogen, Carlsbad, CA) according to manufacturer's instructions. After overnight treatment the serum free EMEM was removed and replaced with complete EMEM. Transfected and un-transfected cells were treated with 1 ug of Dose 2, Dose 3, Dose 4 and Dose 5 each week on Day 8 of each treatment. Un-transfected cells were treated with miRNAs served as control to normalize the effect of miRNAs in transfected MDCK cells with H1N1 and H3N2. All experiments were run in triplicates.

3.9 H1N1 and H3N2 Localization by Immunostaining in Transfected and Untransfected MDCK Cells Treated with and without 10 Potential Identified miRNAs

On day 11 of transfection and on day 7 of each treatment with ten potential miRNAs, MDCK cells transfected with H1N1 and H3N2, un-transfected MDCK cells, MDCK cells only treated with miRNAs and MDCK cells transfected with H1N1 and H3N2 and treated with ten potential miRNAs were washed with 1X PBS, trypsinized, and centrifuged, and the cell pellets were re-suspended in complete EMEM. The cells were added to their respective labeled 8 well

chamber slides (Cat # T-2820-8, Nunc® Lab-Tek® II Glass Chamber Slide™, ThermoFisher Scientific, Wilmington, DE) and were checked for red fluorescence for Cy3 to check the efficacy of penetration of miRNAs tagged with polyarginine peptide and Cy3 and green fluorescence to see the efficiency of transfection and H1N1 and H3N2 localization with Influenza A NP (Nucleoprotein) FITC labeled antibody (Cat # PA1-41071, ThermoFisher Scientific, Wilmington, DE) as described previously [7]. Each week, cells were passaged, preserved in recovery cell culture freezing medium (Cat # 12648-010, Life Technologies Carlsbad, CA), stored for RNA isolation at −80 °C and used for Cy3 fluorescence and H1N1 and H3N2 localization. All experiments were run in triplicates.

3.10 Detection of H1N1 and H3N2 Load in Transfected, Untransfected, and Treated MDCK Cells by qRT- PCR

The iTaq Universal SYBR Green One-Step Kit (Cat # 172-5151, BIO-RAD, Hercules, CA) was used to perform the qRT-PCR to detect the H1N1 and H3N2 load after transection in transfected and un-transfected MDCK cells, and in transfected, un-transfected and treated MDCK cells after treatment. Master mix consisted of 2 X SYBER Green RT-PCR reaction mix of H1N1 and H3N2 primers (HA and NA regions) (Table 1), or β-actin primers (Forward β-actin: 5′-TGGTGATGGAGGAGGTTTAGTAAG- 3′ and Reverse β-actin: 5′-AACCAATAAAACCTACTCCTCCCTTAA-3′) [8]. One-Step RT-PCR was performed according to manufacturer's instructions. β-actin, a house-keeping gene (HKG), was used to obtain a normalized Ct value for both experimental and control samples. Expression of H1N1 and H3N2 was determined for the normalized experimental samples to their respective controls using $2^{(-\Delta\Delta Ct)}$ method. All experiments were run in triplicate.

4.0 RESULTS AND DISCUSSION

4.1 Selection of 50 Nasal Washes

Out of 128 nasal wash samples, 50 samples were selected for the study. The 50 samples included, 10 male and 10 female positive for H1N1 (Table 6), 10 male and 10 female positive for H3N2 (Table 7) and five male and five female negative for both H1N1 and H3N2 (Table 8).

Table 5. Twenty Nasal Wash Samples Positive for H1N1 Using In-House Primers

Male H1N1 Nasal Wash Samples		Female H1N1 Nasal Wash Samples	
Sample Number	**DOD ID**	**Sample Number**	**DOD ID**
1	M19	1	F20 H1N1 A
2	M23	2	F20 H1N1 B
3	M29	3	F21 H1N1
s4	M33 H1N1	4	F26 H1N1 A
5	M38	5	F26 H1N1 B
6	M41	6	F27 H1N1
7	2 H1N1 (M44)	7	10 H1N1 (F28)
8	M49 H1N1	8	3 H1N1 (F30)
9	M50 H1N1	9	F32 H1N1
10	M62	10	8 H1N1 (F37)

Nasal wash samples obtained from 10 male and 10 female participants between ages 18-65 years, tested positive for the influenza A subtype H1N1.

Table 6. Twenty Nasal Wash Samples Positive for H3N2 Using In-House Primers

Male H3N2 Nasal Wash Samples		Female H3N2 Nasal Wash Samples	
Sample Number	**DOD ID**	**Sample Number**	**DOD ID**
1	23 H3N2 (M20)	1	25 H3N2 (F18)
2	9 H3N2 (M24)	2	F19 H3N2
3	11 H3N2 (M41)	3	1 H3N2 (F24)
4	2 H3N2 (M42)	4	F26 H3N2
5	4 H3N2 (M44)	5	10 H3N2 (F28)
6	M45 H3N2	6	5 H3N2 (F31)
7	M51 H3N2	7	F33 H3N2
8	19 H3N2 (M52)	8	6 H3N2 (F38)
9	M53 H3N2	9	F55 H3N2
10	21 H3N2 (M61)	10	F57 H3N2

Nasal wash samples obtained from 10 male and 10 female participants between ages 18-65 years, tested positive for the influenza A subtype H3N2.

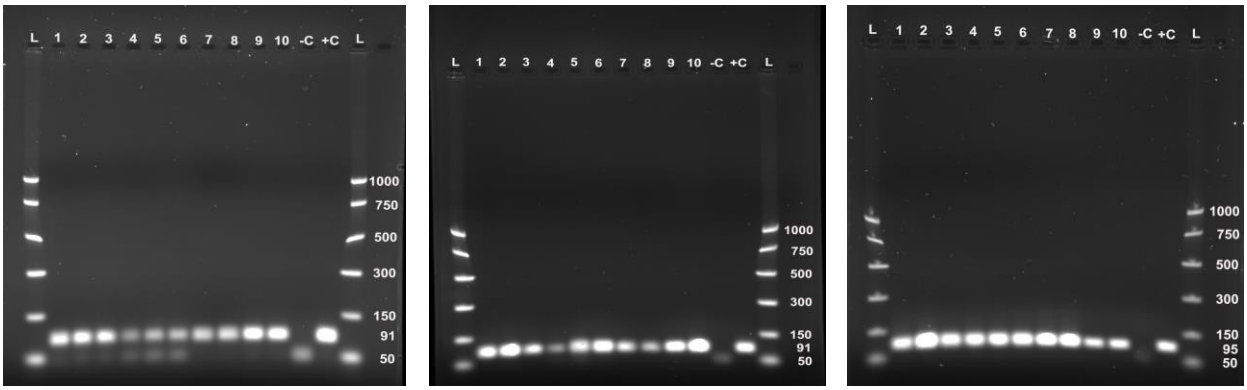
Table 7. Ten Nasal Wash Samples Negative for H1N1 and H3N2, and Two Positive Controls: Isolate H1N1 and Isolate H3N2 Positive for H1N1 and H3N2, Using In-House Primers

Male Negative Nasal Wash Samples		Female Negative Nasal Wash Samples	
Sample Number	**DOD ID**	**Sample Number**	**DOD ID**
1	2 Neg (M26)	1	F25 Neg
2	M27 Neg	2	3 Neg (F29)
3	M35 Neg	3	F33 Neg
4	1 Neg (M37)	4	F41 Neg
5	M45 Neg	5	F49 Neg
Positive Controls for H1N1 and H3N2			
1	Isolate H1N1		
2	Isolate H3N2		

Nasal wash samples obtained from 5 male and 5 female participants between the ages of 18-65 years, tested negative for the influenza A subtype H1N1 and H3N2. The two positive controls: Isolate H1N1 and Isolate H3N2 tested positive for the influenza A subtype H1N1 and H3N2.

4.2 Confirmation of Negative Controls, and H1N1 and H3N2 Positive Nasal Wash Samples by Gel Electrophoresis

H1N1 and H3N2 showed amplifications of 91 bp and 95 bp, respectively. Figures 1 and 2 show the confirmation of all 52 samples, separated by the subtype and sex using the in-house primers. Similar results were also seen with DoD primers and probes of 100 bp and 150 bp amplification for H1N1 and H3N2, respectively (data not shown). A 1 kb (1000-50 bp, Cat # G3161, Promega, Madison, WI) PCR marker was used in gel electrophoresis.

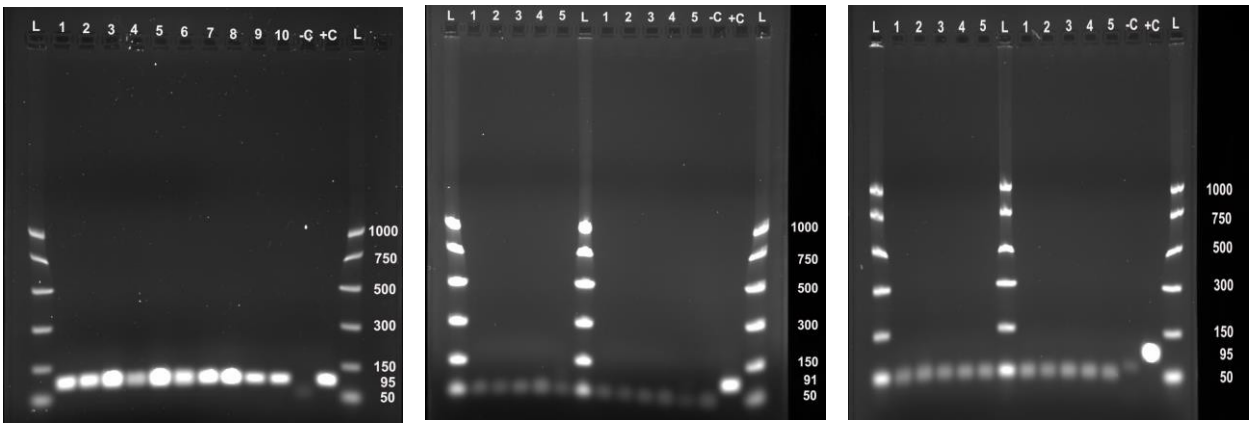


A. Male Nasal Wash Samples Positive for H1N1. The L lanes represent 1 kb marker (1000-50 bp). Lane 1-10 represent 10 male nasal wash samples positive for H1N1, showing amplification product of 91 bp. Positive H1N1 control also shows an amplification product of 91 bp and negative control shows no amplification product for H1N1.

B. Female Nasal Wash Samples Positive for H1N1. The L lanes represent 1 kb marker (1000-50 bp). Lane 1-10 represent 10 female nasal wash samples positive for H1N1, showing amplification product of 91 bp. Positive H1N1 control also shows an amplification product of 91 bp and negative control shows no amplification product for H1N1.

C. Male Nasal Wash Samples Positive for H3N2. The L lanes represent 1 kb marker (1000-50 bp). Lane 1-10 represent 10 male nasal wash samples positive for H3N2, showing amplification product of 95 bp. Positive H3N2 control also shows an amplification product of 95 bp and negative control shows no amplification product for H3N2.

Figure 1. Gel Electrophoresis of Selected Nasal Wash Samples Positive for H1N1 and H3N2. Images of each gel correspond to the nasal wash samples in Tables 5 and 6, confirming that the nasal wash samples and positive control were positive for H1N1 and H3N2.



D. Female Nasal Wash Samples Positive for H3N2. The L lanes represent 1 kb marker (1000-50 bp). Lane 1-10 represent 10 female nasal wash samples positive for H3N2, showing amplification product of 95 bp. Positive H3N2 control also shows an amplification product of 95 bp and negative control shows no amplification product for H3N2.

E. Male and Female Negative for H1N1. The L wells represent 1 kb marker (1000-50 bp). Left lane 1-5 and Right lane 1-5 represent male and female nasal wash samples, respectively, negative for H1N1. Positive H1N1 control shows an amplification product of 91 bp and negative control shows no amplification product for H1N1.

F. Male and Female Negative for H3N2. The L wells represent 1 kb marker (1000-50 bp). Left lane 1-5 and Right lane 1-5 represent male and female nasal wash samples, respectively, negative for H3N2. Positive H3N2 control shows an amplification product of 95 bp and negative control shows no amplification product for H3N2.

Figure 2. Gel Electrophoresis of Selected Nasal Wash Samples Positive for H3N2 and Negative for H1N1 and H3N2. Images of each gel corresponds to the nasal wash samples in Tables 6 and 7, confirming that the nasal wash samples and positive control were positive for H3N2 and the controls were all negative for H1N1 and H3N2.

4.3 miRNA Profiling and Analysis

Table 8 shows five down-regulated and five up-regulated human miRNAs (hsa-miRs) out of 2,402 hsa-miRs using Human miRNome, miRBase V21. Table 6 also shows the fold regulation, p-values and composite ranking for ten potential identified miRNAs identified for each subtype: H1N1 and H3N2. Negative numbers display the downregulated miRNAs and positive numbers display the up-regulated miRNAs.

Table 8. Common MicroRNAs Expressed in H1N1 and H3N2 Infected Nasal Wash Samples

MiRNA No.	MiRNA	Accession Number	Sequence	Base Pairs	Fold vs H1N1	Fold vs H3N2	p-value vs H1N1	p- value vs H3N2	Composite Rank
1	hsa-miR-1233-5p	MIMAT0022943	AGUGGGAGGCCAGGGCACGGCA	22 bp	-2.76	-25.64	0.0015	0.0083	-1
2	hsa-miR-7847-3p	MIMAT0030422	CGUGGAGGACGAGGAGGAGGC	21 bp	-13.61	-2.84	0.0003	0.0002	-2
3	hsa-miR-6875-3p	MIMAT0027651	AUUCUUCCUGCCCUGGCUCCAU	22 bp	-8.2314	-6.97	0.0050	0	-3
4	hsa-miR-6832-5p	MIMAT0027564	AGUAGAGAGGAAAAGUUAGGGUC	23 bp	-7.25	-6.86	0.0220	0.0017	-4
5	hsa-miR-7114-3p	MIMAT0028126	UGACCCACCCCUCUCCACCAG	21 bp	-8.87	-5.11	0.0090	0.0000	-5
6	hsa-miR-3154	MIMAT0015028	CAGAAGGGGAGUUGGGAGCAGA	22 bp	11.11	12.33	0.0060	0.0019	1
7	hsa-miR-3173-3p	MIMAT0015048	AAAGGAGGAAAUAGGCAGGCCA	22 bp	3.72	9.76	0.0395	0.0001	2
8	hsa-let-7e-5p	MIMAT0000066	UGAGGUAGGAGGUUGUAUAGUU	22 bp	10.01	3.42	0	0.0124	3
9	hsa-miR-3691-5p	MIMAT0018120	AGUGGAUGAUGGAGACUCGGUAC	23 bp	2.86	8.97	0.0015	0.0107	4
10	hsa-miR-101-5p	MIMAT0004513	CAGUUAUCACAGUGCUGAUGCU	22 bp	2.3	7.30	0.0036	0.0321	5

Note: Indicated are the miRNA sequences, showing the fold change and p value of each respective miRNA sequence vs the influenza A subtypes. Fold expression and p value for each miRNA were used to generate composite ranking for each miRNA. Fold regulation and p value were provided by Qiagen Genomic Services (Qiagen, Frederick, MD). Composite ranking indicates the down-regulated and up-regulated expression of the miRNAs. The top five miRNAs are down-regulated and the bottom five miRNAs are up-regulated for both H1N1 and H3N2.

4.4 Up-Regulated miRNAs Reduces Cytopathic Effect in H1N1 and H3N2 Transfected and Treated MDCK Cells

CPE increased with incubation time in MDCK cells transfected only with H1N1 and H3N2 (data not shown). It was also observed that the CPE formation increased in MDCK cells transfected with H1N1 and H3N2 and treated with 5 common down-regulated miRNAs after Dose 3 treatment (data not shown). The CPE formation decreased in MDCK cells transfected with H1N1 and H3N2 and treated with 5 common up-regulated miRNAs after Dose 3 treatment (data not shown). It was also observed that the un-transfected and treated MDCK cells showed no CPE formation (data not shown). These results clearly indicate that the 5 common up-regulated miRNAs were effective in reducing CPE formation in MDCK cells.

4.5 Up-Regulated miRNAs Reduces H1N1 and H3N2 Infection in Treated MDCK Cells

MDCK cells transfected with only H1N1 and H3N2 showed an increase in the number of green dot-like structures with incubation time (Figure 3). MDCK cells that were transfected with H1N1 and H3N2 and treated with 5 common down-regulated miRNAs showed an increase in the number of green dot-like structures after Dose 3 treatment (Figure 4: hsa-miR-1233-5p). MDCK cells transfected with H1N1 and H3N2 and treated with 5 common up-regulated miRNAs showed a decrease in the number of green dot-like structures after Dose 3 treatment s (Figure 5: hsa-miR-3154). The immunostaining results indicate that 5 common up-regulated miRNAs treatment is effective in decreasing the localization of both H1N1 and H3N2 in MDCK cells consistent with CPE formation results.

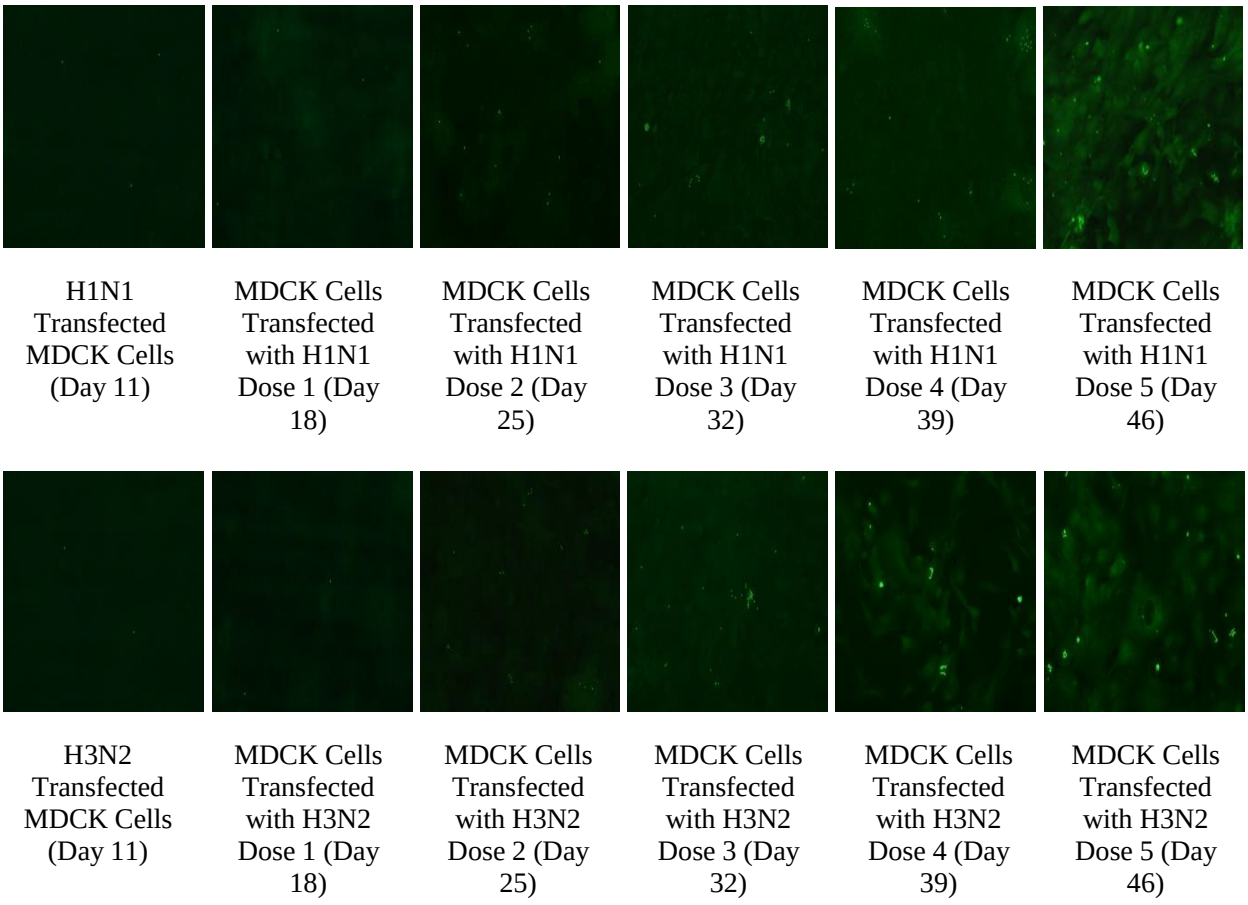
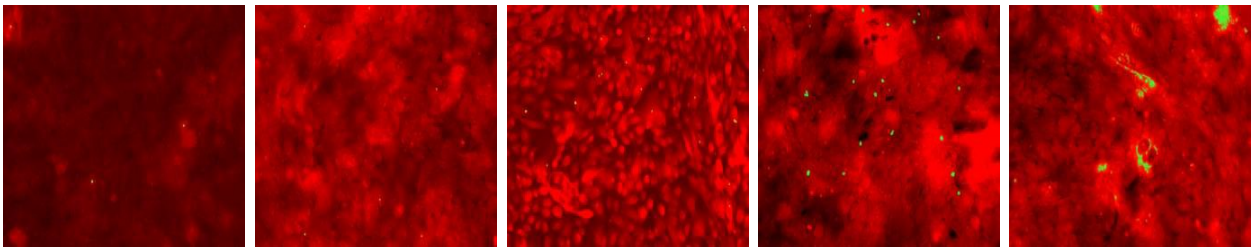
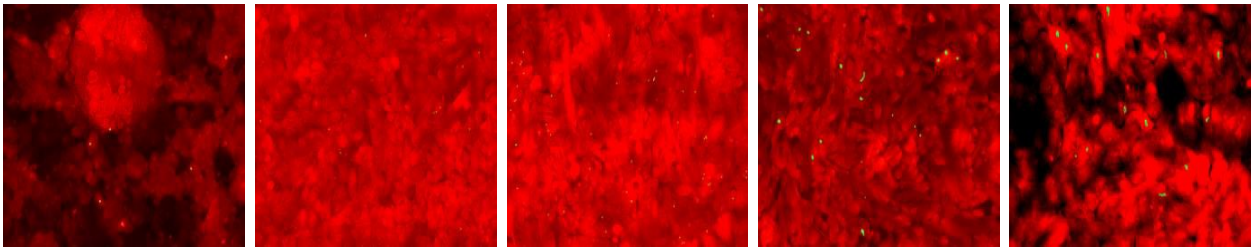


Figure 3. Increase in H1N1 and H3N2 localization in Untreated MDCK cells. Green dot-like structures in untreated MDCK cells transfected with H1N1 and H3N2 increased with incubation time.

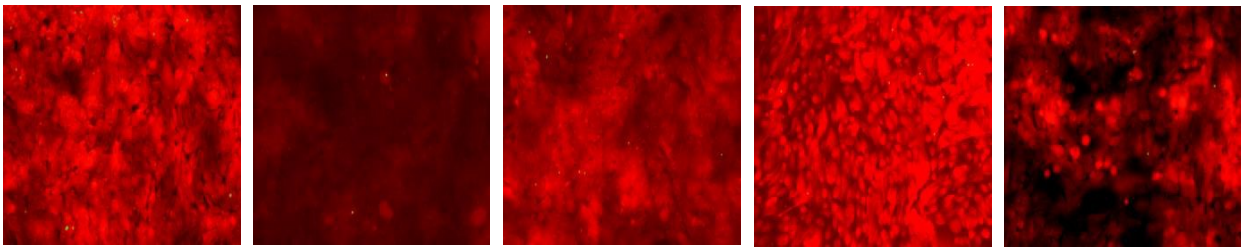


MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-1233-5p (Day 18)	MDCK Cells Transfected with H1N1 and Treated with Dose 2: hsa-miR-1233-5p (Day 25)	MDCK Cells Transfected with H1N1 and Treated with Dose 3: hsa-miR-1233-5p (Day 32)	MDCK Cells Transfected with H1N1 and Treated with Dose 4: hsa-miR-1233-5p (Day 39)	MDCK Cells Transfected with H1N1 and Treated with Dose 5: hsa-miR-1233-5p (Day 46)



MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-1233-5p (Day 18)	MDCK Cells Transfected with H3N2 and Treated with Dose 2: hsa-miR-1233-5p (Day 25)	MDCK Cells Transfected with H3N2 and Treated with Dose 3: hsa-miR-1233-5p (Day 32)	MDCK Cells Transfected with H3N2 and Treated with Dose 4: hsa-miR-1233-5p (Day 39)	MDCK Cells Transfected with H3N2 and Treated with Dose 5: hsa-miR-1233-5p (Day 46)

Figure 4. Increase in H1N1 and H3N2 localization in MDCK cells Treated with hsa-miR-1233-5p. Green dot-like structures in MDCK cells transfected with H1N1 and H3N2 and treated with hsa-miR- 1233-5p increased tremendously after dose 3 treatment when compared to their respective controls (Figure 3).



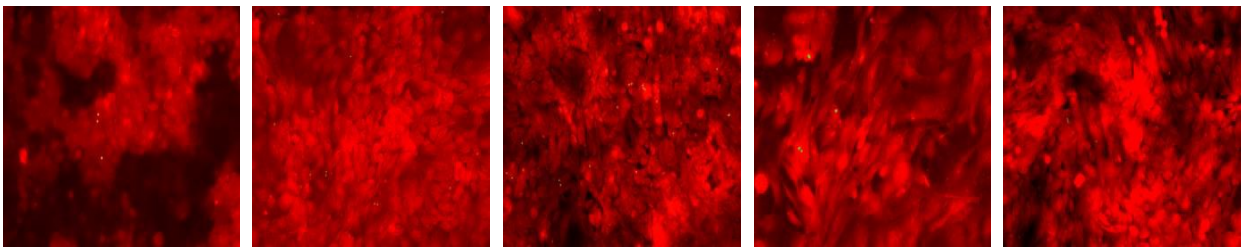
MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-3154 (Day 18)

MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-3154 (Day 25)

MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-3154 (Day 32)

MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-3154 (Day 39)

MDCK Cells Transfected with H1N1 and Treated with Dose 1: hsa-miR-3154 (Day 46)



MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-3154 (Day 18)

MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-3154 (Day 25)

MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-3154 (Day 32)

MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-3154 (Day 39)

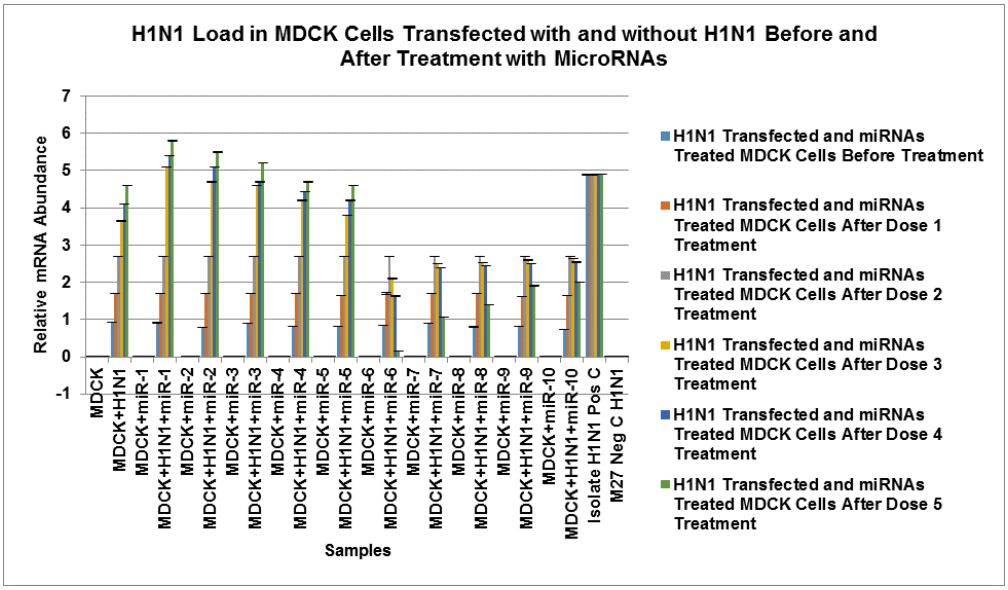
MDCK Cells Transfected with H3N2 and Treated with Dose 1: hsa-miR-3154 (Day 46)

Figure 5. Decrease in H1N1 and H3N2 localization in MDCK cells Treated with hsa-miR-3154. Green dot-like structures in MDCK cells transfected with H1N1 and H3N2 and treated with hsa-miR- 3154 decreased tremendously after dose 3 treatment when compared to their respective controls (Figure 3).

4.6 Up-Regulated miRNAs Decreases H1N1 and H3N2 Load

qRT-PCR was used to evaluate the effect of 5 common down-regulated miRNAs and 5 common up-regulated miRNAs on H1N1 and H3N2 load. MDCK cells transfected with only H1N1 and H3N2 showed a gradual increase in H1N1 and H3N2 load over the course of time. MDCK cells that were transfected with H1N1 and H3N2 and treated with 5 common down-regulated miRNAs showed an increase in both H1N1 and H3N2 load after Dose 3 treatment (Figure 6). Whereas MDCK cells that were transfected with H1N1 and H3N2 and treated with 5 common up-regulated miRNAs showed a decrease in both H1N1 and H3N2 load after Dose 3 treatment (Figure 7). It was also observed that hsa- miR-1233-5p that was down-regulated -2.76 fold and -25.64 fold by microarray profile for H1N1 and H3N2, respectively, increased the H1N1 and H3N2 load. Whereas, hsa-miR-3154 that was up-regulated 11.11 fold and 12.33 fold by microarray profile for H1N1 and H3N2, respectively lowered the H1N1 and H3N2 load. The decrease in H1N1 and H3N2 load for 5 common up-regulated miRNAs is consistent with

decrease in CPE formation and with immunostaining showing reduced localization of H1N1 and H3N2 with Influenza A NP FITC labeled antibody.



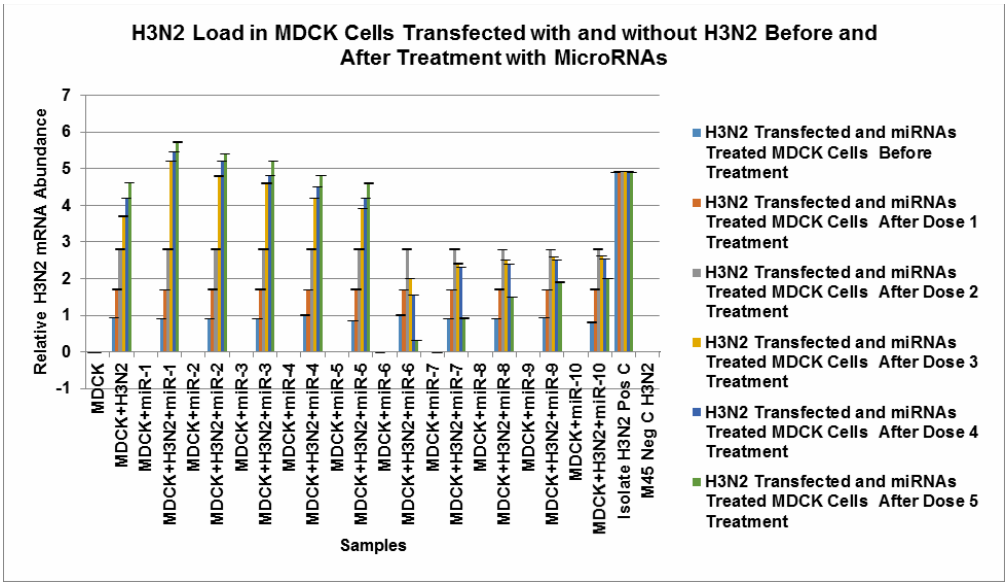


Figure 6. Effect of 5 common down-regulated miRNAs and 5 common up-regulated miRNAs on H1N1 and H3N2 load over 5 weeks in MDCK Cells by qRT-PCR. Data are shown as $\log_{10}$ ratio and reflect the expression of H1N1 and H3N2 load in MDCK cells transfected with H1N1 and H3N2 and treated with 5 common down-regulated and up-regulated potential identified miRNAs. Expression of H1N1 and H3N2 were normalized using the β-actin expression level as a housekeeping gene. The normalized experimental values were analyzed with their respective normalized control values using the $2^{(-\Delta\Delta Ct)}$ method, giving relative mRNA abundance of H1N1 and H3N2. Upper chart shows that MDCK cells transfected with H1N1 and H3N2 and treated with 5 common down-regulated miRNAs: hsa-miR-1233-5p, hsa-miR-7847-3p, hsa-miR-6875-3p, hsa-miR-6832-5p and hsa-miR-7114-3p show an increase in both H1N1 and H3N2 load after Dose 3 treatment, with hsa-miR-1233-5p showing more pronounced increase in both H1N1 and H3N2 load. Whereas, lower chart shows that MDCK cells transfected with H1N1 and H3N2 and treated with 5 common up-regulated miRNAs: hsa-miR-3154, hsa- miR-3173-3p, hsa-let-7e-5p, hsa-miR-3691-5p and hsa-miR-101-5p show a decrease in both H1N1 and H3N2 load after Dose 3 treatment, with hsa-miR-3154 showing more pronounced decrease in both H1N1 and H3N2 load.

5.0 CONCLUSIONS

In conclusion, our present in vitro findings suggests that the polyarginine peptide carrying the targeted miRNA was successful in penetrating through the host MDCK cells, and 5 common potential identified up-regulated miRNAs from infected influenza A subtypes H1N1 and H3N2 nasal washes: hsa- miR-3154, hsa-miR-3173-3p, hsa-let-7e-5p, hsa-miR-3691-5p and hsa-miR-101-5p lowered both H1N1 and H3N2 load, with hsa-miR-3154 showing more pronounced decrease in both H1N1 and H3N2 load. Hence, hsa-miR-3154 may be a potential candidate for treating influenza A subtypes H1N1 and H3N2 infections, and a cocktail of these 5 common potential identified up-regulated miRNAs should be investigated as an alternate for vaccination of influenza A subtypes H1N1 and H3N2.

6.0 REFERENCES

1. Suzuki Y. 2005. Sialobiology of influenza: molecular mechanism of host range variation of influenza viruses. Biol Pharm Bull. 2005; 28(3):399–408.
2. World Health Organization. 2009. Influenza Seasonal: World Health Organization, WHO fact sheet 211. [Accessed 21 Feb 2019]. Available from http://www.who.int/mediacentre/factsheets/fs211/en/.
3. Lamb RA, Choppin PW. The gene structure and replication of influenza virus. Ann Rev Biochem. 1985; 52:467–506.
4. Bouvier NM., Palese P. The biology of influenza viruses. Vaccine. 2008; 26(4):D49–53.
5. Jeffery DD, Cohen M, Brooks A, Linton A, Gromadzki, R, et al. Impact of the 2009 influenza (H1N1) pandemicon the United States military health care system. Mil Med. 2013; 178(6):653-658.
6. World Health Organization. 2011. Influenza Surveillance Monitoring: World Health Organization. [Accessed 21 Feb 2019]. Available from http://www.who.int/influenza/surveillance_monitoring/updates/2011_01_14_GIP_surveillance/en/.
7. Noorali S, Sheraz M, Tisdale SS, Dallas SS, Simons LM. Effect of differentially expressed MicroRNAs 602 and 323-5p on Hepatitis C Virus genotype 1b viral load in infected liver cells. J Infect Dis Ther. 2014; 2:138.
8. Cohen, Y., Singer, G., Lavie, O., Dong SM, Beller U, et al. The RASSF1A tumor suppressor gene is commonly inactivated in adenocarcinoma of the uterine cervix. Clin Cancer Res. 2003; 9(8):2981-2984.

LIST OF ABBREVIATIONS AND ACRONYMS

CPE	cytopathic effect
EMEM	Eagle's Minimum Essential Medium
HA	Hemagglutinin
Hsa-miR	human miRNA
MDCK	Madin-Darby Canine Kidney
miRNA	microRNA
NA	neuraminidase
qRT-PCR	quantitative reverse transcription polymerase chain reaction