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TITLE: Capturing Antibiotic-Resistant Ribosomes

PRINCIPAL INVESTIGATOR: Mee-Ngan Frances Yap, Ph.D.

CONTRACTING ORGANIZATION: Saint Louis University

Saint Louis, 63108

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14. ABSTRACT

The universally conserved nucleotide (A2058) of 23S rRNA in all bacterial ribosomes, when methylated (m^6A2058), causes cross-resistance to multiple families of therapeutically important antibiotics. The abundance and essentiality of ribosomes make them an attractive target for the detection of resistant pathogens based on the unique m^6A2058 signature. The goal of this study is to develop immunoreagents that can be used as a rapid diagnostic tool for antibiotic resistant bacteria. They can also be used as a capturing tool to isolate homogenous populations of m^6A2058-ribosomes for structural and biochemical determination, a critical step to delineate the molecular mechanisms of new ribosome-targeting antibiotics. In Year 1, our success in the initial phase (Aims 1-2) has offered a solid proof-of-principle to further improve the immunoreagents that specifically recognize the resistant ribosomes bearing the m^6A2058 modification. In addition to our effort in increasing the specificity, affinity and yield of the available immunoreagents, we will perform an in vitro phage display screen to identify synthetic peptides against the m^6A2058 ribosome as proposed in Aim 3.

15. SUBJECT TERMS
RNA methylation, ribosome, antibiotic resistance, macrolide

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1. INTRODUCTION

Dimethylation of a universally conserved adenine, A2058, in bacterial rRNA causes cross-resistance against all three critically important families of antibiotics (**m**acrolides, **l**incosamides, and **s**treptogramins (MLS))(1). A2058 dimethylation (hereafter called m^6A2058) occludes MLS from the ribosome, thereby allowing normal protein biosynthesis and bacterial growth (2). The RNA methylation enzyme *ermB* is widespread in almost all ESKAPE (***E**nterococcus faecium, **S**taphylococcus aureuus, **K**lebsiella pneumonia, **A**cinetobacter baumannii, **P**seudomonas aeruginosa, and **E**nterobacter species*) pathogens, which are the leading causes of hospital-acquired infections (HAI). Erm-mediated MLS resistance often represent up to 60-98% of the pathogenic MLS-resistant clinical isolates, a percentage that surpasses MLS resistance caused by drug inactivation and efflux mechanisms combined.

Overcoming *ermB*-mediated MLS resistance has been hampered by the inability to isolate homogeneous population of m^6A2058-ribosomes for structural and biophysical kinetic studies of MLS binding. In addition, detection of ErmB-resistant strains in clinical settings often requires blood culture followed by PCR amplification of the *erm* genes or by antibiotic susceptibility tests, both of which are time consuming. The latter also suffers from sensitivity issues. Ribosomes represent more than one-third of the bacterial biomass, and approximately two-thirds of ribosomes are composed of rRNA. This project seeks to develop a bifunctional tool capable of pulling down m^6A2058 ribosomes from crude bacterial lysates with the ability to detect the resistant ribosomes in blood without conventional microbial culture. To this end, we were able to obtain antibody-RNA conjugate and natural antibody that exhibit high specificity towards m^6A2058 RNA.

2. KEYWORDS

RNA methylation, macrolide, antibiotic resistance, ribosome, m^6A, ESKAPE pathogen, translational regulation, infectious diseases, bacterial pathogenesis.

3. ACCOMPLISHMENTS

3.1. What were the major goals of the project?

We proposed to use a three-pronged approach to develop antibody and/or aptamer that specifically binds to $m^6{}_2$A2058 ribosome. Specific goals are:
Aim 1. To engineer and antibody-oligo conjugate specific for $m^6{}_2$A2058 ribosomes.
Aim 2. To generate natural antibodies using an RNA-carrier conjugate.
Aim 3. To generate synthetic peptide binders using phage display.

3.2. What was accomplished under these goals?

Aim 1. To engineer and antibody-oligo conjugate specific for $m^6{}_2$A2058 ribosomes.

We have significantly improved the specificity of a commercially available monoclonal antibody (Synaptic systems) that cross-reacts with m^6-adenosine (m^6A). First, we performed enzyme-linked immunosorbent assay (ELISA) experiment to quantitate the amount of m^6A methylation in the total RNA isolated from the *Staphylococcus aureus ermB*-deficient (*ermB*$^-$), *ermB*-proficient (*ermB*$^+$), and a catalytically inactive Y103A allele ((*ermB*^{Y103A}). As shown in Figure 1A-1B, we found that RNA from the *ermB*$^+$ strain is > 3 times more methylated than the *ermB*$^-$ and *ermB*^{Y103A} strains, presumably due to specific methylation of the A2058 nucleotide in the 23S rRNA; whereas the 12-13% methylation in the *ermB*$^-$ and *ermB*^{Y103A} strains were attributed to background m^6A in the other five non-A2058 sites. These data indicate that the monoclonal antibody has the ability to enrich the RNAs bearing the m^6A2058 modification but

cannot discriminate m^{6}A2058 from other m^{6}A-RNA molecules, e.g. m^{6}A1518, m^{6}A1519 in the 16S rRNA and m^{6}A1618, m^{6}A2030, and m^{6}A2503 in the 23S rRNA.

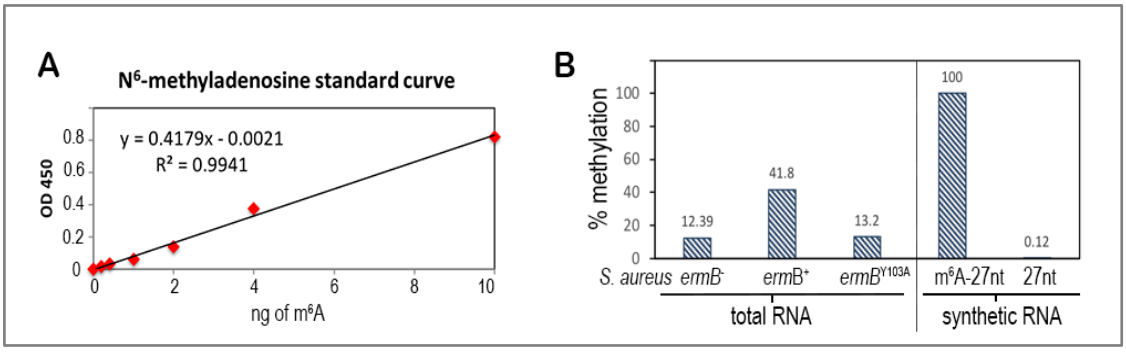

Figure 1. Detection of m^{6}A2058 modified RNA by ELISA. **(A)** Standard curve using the N^6-methyladenosine (m^{6}A) as the reference. **(B)** Degree of A2058 N^6-methylation upon ErmB expression. Two hundred nano-grams of total RNA isolated from the *Staphylococcus aureus ermB*-deficient (*ermB*$^-$), *ermB*-proficient (*ermB*$^+$), and a catalytically inactive Y103A ((*ermB*^{Y103A}) strains were used in each reaction. Unmodified or m^{6}A2058-modified synthetic RNAs of 27-nt was used as a negative and positive control, respectively. RNA targets were immobilized to a 96-well microplate and subsequently captured with an HRP-conjugated anti-m^{6}A (Synaptic LLC). The amount of m^{6}A modification was determined using the standard curve in panel (A). The percentage of m^{6}A is calculated relative to the m^{6}A-27 nt control.

To improve the specificity of the antibody against m^{6}A2058, we conjugated the antibody with a 12-mer anti-sense oligonucleotide against either the upstream (5'-GCGACAGGACGG) or the downstream region (5'-AAGACCCCGUGG) of m^{6}A2058 by the Solulink's HyNic/4FB bioconjugation linkage system (TriLink). This system allows the removal of the unconjugated HyNic-antibody and 4FB-oligonucleotides without laborious purification steps. We tested the specificity of the antibody-oligo adducts by North-Western hybridization. Total RNA from *S. aureus* lacking an RNA methyltransferase gene *ermB* (Δ*ermB*) or containing a catalytically inactive *ermB*^{Y103A} and the wild type *ermB* were resolved on a denaturing agarose gel, transferred to a nylon membrane, and hybridized with the antibody-oligo conjugates (Figure 2). We found that the conjugate harboring the downstream antisense oligo successfully reacts to the m^{6}A2058 in the 23S rRNA but cannot discriminate m^{6}A epigenetic marks from that in the 16S rRNA. Our ongoing work include extending the oligo lengths to 20 nucleotides to better differentiate the m^{6}A in the 16S rRNA in a sequence-specific manner.

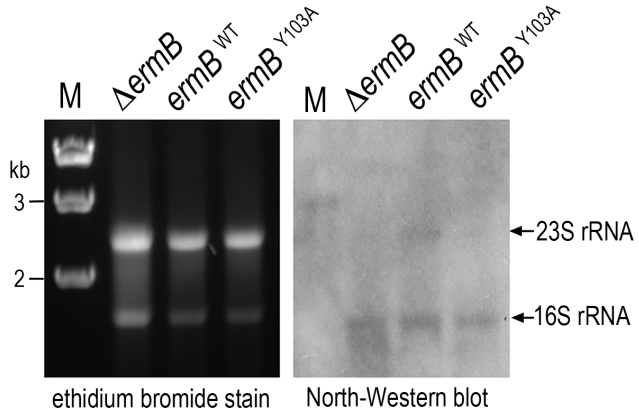

Figure 2. Northwestern blot confirming the specificity of the antibody-oligo conjugate. (Left) Ethidium bromide stained denaturing gel showing the 23S rRNA and 16S rRNA of *S. aureus* strains. Two-micrograms of DNAse-treated RNAs were loaded per lane. **(Right)** Recognition of the antibody-oligo conjugate (1:50 dilutions) specifically with the 23S RNA in the *ermB*- expressing strain, but not the catalytically dead mutant Y103A or a strain lacking *ermB*. The antibody conjugate also cross-reacts with the m^{6}A marks in the 16S rRNA.

Aim 2. To generate natural antibodies using an RNA-carrier conjugate.

We had conjugated a 27 nt synthetic RNA bearing the m^6A2058 modification with a high-molecular weight carrier weight carrier **k**eyhole **l**impet **h**emocyanin (KLH) using Imject™ Mariculture KLH conjugation kit (Thermo Scientific). We covalently linked to covalently link the 5' amine-containing m^6A-RNA (IDT DNA) via the amine-reactive NHS-ester, followed by size exclusion chromatography to remove uncoupled RNA or KLH (3). This conjugate was used to raised antibodies in rabbits, guinea pigs and rats. We found this RNA conjugate was only able to elicit proper immune response in rabbits but not in other animals (see "notes on animal use"). Our dot blot analysis using purified RNA from different *S. aureus* backgrounds showed that the antibody generated from rabbit hosts is highly specific to *ermB*-producing strains but not to the strains with unmodified-A2058 RNA (Figure 3). We are in the process of affinity purify the IgG in order to test its affinity to the assembled ribosomes.

Notes on the use of animals: No fund from this award has been used for animal work as of July 15, 2019. Antibody production was an ongoing work before the notice of award in 2018. A commercial vendor (Josman, LLC) conducted the experiments and the service fees were paid from the principle investigator's university startup fund.

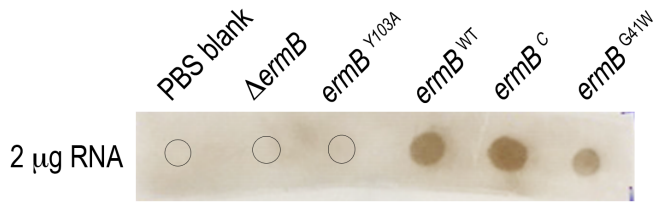

Figure 3. Dot blot demonstrating the specificity of anti-rabbit anti-m^6A-RNA. Purified total RNAs from different S. aureus strains were hybridized with 1:500 dilutions of antisera. The antibody does not recognize RNA from a strain lacking *ermB* (Δ*ermB*) and catalytically inactive $ermB^{Y103A}$ but strongly reacts with that of strains carrying the wild-type *ermB*, a constituitively expressed *ermB* ($ermB^{C}$) and a benign mutation in *ermB* ($ermB^{G41W}$).

Summary: We have met the milestones proposed in the first year SOW. Although additional experiments are necessary to improve the quality and quantity of the developed immunoreagents, we obtained highly specific immunoreagents (antibody-RNA conjugate and antibody) against the m^6A2058-ribosomes.

3.3. What opportunities for training and professional development has the project provided?

This project has supported a B.S. degree research technician (Ms. Kathryn Shields) and has partially funded one postdoctoral fellow (Dr. Arnab Basu). Dr. Basu had assisted Ms. Shields in performing experiments and providing guidance in data collection and interpretation. Dr. Basu has gained new knowledge in ribosome modification and enzymology. In the past year Dr. Basu had interviewed several faculty positions in his home country India. The lab had offered feedback on this presentations and application package. Ms. Shields was not familiar with Staphylococcal genetics and protein and RNA chemistry prior to joining the team. She has since then broadened her technical and conceptual skills in these areas through her efforts in creating and confirming an antibody that specifically binds to the $m^6{}_2A2058$ ribosomes. Ms. Shields has a manuscript in the pipeline, in which she receives writing guidance from the PI. Both trainees had presented their work in the national and regional conferences.

3.4. How were the results disseminated to communities of interest?

Nothing to report.

3.5. What do you plan to do during the next reporting period to accomplish the goals?

In the remaining 6 months, we plan to complete the proposed Aim 3 by performing in vitro phage display screening to identify peptide binders that specifically interact with the $m^6{}_2$A2058 ribosomes (4, 5). Our ongoing effort includes increasing the yield and specificity of the antibody-oligo conjugate (Aim 1) and affinity purification of the naturally produced antibody (Aim 2).

4. IMPACT

4.1. What was the impact on the development of the principle discipline(s) of the project?

There is an unmet need for highly specific antibodies for resistant m^6A2058-ribosomes. Success in the initial phase (Aims 1-2) of this long-term project has offered a solid proof-of-principle to develop the immunoreagents required for the detection of other RNA modifications at single nucleotide resolution. The immediate impact of this proposal includes the mechanistic validation of the new macrolides. The m^6A2058 modified regions are evolutionarily conserved across all resistant bacterial species; thus, the antibody-based diagnostic kits will be able to detect one or more bacterial pathogens that link the resistance profiles to the abundance of m^6A2058-ribosomes. These tools will also have a long-lasting impact on patient care by providing a sensitive, specific and adaptable assay for pathogen detection, thereby allowing timely treatment decisions.

4.2. What was the impact on other disciplines?

The availability of the proposed immunoreagents will significantly advance both applied (clinical diagnostics) and basic RNA and ribosome biology as a whole.

4.3. What was the impact on technology transfer?

The developed immunoreagents potentially are patentable for commercial use in the future.

4.4. What was the impact on society beyond science and technology?

Presentations of the findings produced from this project raised public awareness about antimicrobial resistance and its counter strategies.

5. CHANGES/PROBLEMS

5.1. Changes in approach and reasons for change.

No significant changes in approach.

5.2. Actual or anticipated problems or delays and actions or plans to resolve them.

The lab will be relocating from the Saint Louis University to Northwestern University in Chicago in July 2019. We expected some experimental interruptions because the new laboratory will not be fully operated in the first few weeks, and the current research technician on the project will not join Northwestern University due to family obligation in St. Louis. These potential delays will be resolved by the following: (1) all of the key instrumentation relevant to this project is readily available at the new institution, (2) the substitute of the research technician, a postdoctoral fellow, is experienced in ribosome biology, (3) the PI has been relieved from teaching to focus on new lab and research, and finally (4) the PI has obtained the approvals of Institutional Biosafety and Chemical Safety Protocols to allow initiation of the wet bench research.

5.3. Changes that had a significant impact on expenditures.

Nothing to report.

5.4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

5.4.1 Significant changes in use or care of human subjects.
Not applicable.

5.4.2. Significant changes in use or care of vertebrate animals.
Nothing to report.

5.4.3. Significant changes in use of biohazards and/or select agents.
Nothing to report. Staphylococcus aureus is the primary organism.

6. PRODUCTS

6.1. Publications, conference papers, and presentations.

6.1.1. Books or non-periodical, one-time publications.
Not applicable

6.1.2. Other publications, conference papers, and presentations.

Publication
Shields, K., and Yap, M.-N. F. (2019) Selective translation and antibiotic vulnerability of methylated ribosomes. *Under review*.

Invited seminars
- M.-N. F. Yap (March 2019). "Ribosome silencing as a bacterial survival strategy". Department of Microbiology. The Ohio State University, Columbus, OH, USA.
- M.-N. F. Yap (April 2019). "Stress management by ribosome silencing". Center for Infectious Diseases, Tsinghua University School of Medicine, Beijing, China.
- M.-N. F. Yap (Jan 2019)." Ribosome silencing in bacterial pathogenesis". Bacteriology Laboratory, Wadsworth Center, Albany, NY, USA.

Conference Plenary talks
- M.-N. F. Yap (Jan 2019) "Regulation of ribosome silencing". Ribosome Structure and Function 2019, Merida, Mexico.

Conference abstracts
- Basu, A., Shields, K., and Yap, M.-N. F. (2018) 25th Annual Microbial Pathogenesis Conference. "Ribosome inactivation in Staphylococcal pathogenesis". University of Iowa, Iowa City, IA, USA.

- Basu, A., and Yap, M.-N. F. (2018) Gordon conference in Microbial Stress Response. "Thermal and nutritional control of ribosome hibernation". Mount Holyoke College, MA, USA.

6.2. Website(s) or other internet site(s)
Nothing to report.

6.3. Technologies or techniques.
Nothing to report.

6.4. Inventions, patent application, and/or licenses
Nothing to report.

6.5. Other products.
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

7.1. what individuals have worked on the project?

Name	M.-N. Frances Yap, Ph.D.
Project role	Principle Investigator
Effort (months worked)	1.8
Contribution	Dr. Yap was responsible for the overall coordination and supervision of this study. This includes experimental design, training, analyzing data and preparing for dissemination of the results.
Funding support	This grant, NIH GM121359, Saint Louis University, Edward Mallinckrodt, Jr Foundation.

Name	Arnab Basu, Ph.D.
Project role	Postdoctoral Fellow
Effort (months worked)	2.8
Contribution	Dr. Basu trained Ms. Shields in her first 3 months of employment. Dr. Basu provided technical guidance, conceptual input and data interpretation.
Funding support	This grant, NIH GM121359

Name	Kathryn Shields, B.Sc.
Project role	Research Technician
Effort (months worked)	12
Contribution	Ms. Shields performed work in identifying, modifying and testing the specificity of antibodies that recognizes $m^6{}_2A$ epigenetic marks
Funding support	This grant

7.2. Has there been a change in the active other support of the PI(s) or key personnel since the last reporting period?
No

7.3. What other organizations were involved as partners?
Not applicable. Saint Louis University is the sole performing site.

8. SPECIAL REPORTING REQUIREMENTS
Not applicable

9. APPENDICES
See revised “Statement of work (SOW)”

REFERENCES

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2. **Dzyubak E, Yap MN.** 2016. The expression of antibiotic resistance methyltransferase correlates with mRNA stability independently of ribosome stalling. Antimicrob Agents Chemother **60:**7178-7188. PMCID: PMC5118997
3. **Ye JD, Tereshko V, Frederiksen JK, Koide A, Fellouse FA, Sidhu SS, Koide S, Kossiakoff AA, Piccirilli JA.** 2008. Synthetic antibodies for specific recognition and crystallization of structured RNA. Proc Natl Acad Sci U S A **105:**82-87.
4. **Laird-Offringa IA, Belasco JG.** 1996. In vitro genetic analysis of RNA-binding proteins using phage display libraries. Methods Enzymol **267:**149-168.
5. **Mayer G, Famulok M.** 2009. *In vitro* selection of conformational probes for riboswitches. Methods Mol Biol **540:**291-300.

STATEMENT OF WORK – Month/Day/Year
PROPOSED START DATE 07/01/2019

Site 1: July 2018-June 30, 2019[A]
Saint Louis University [A]
1100 South Grand Blvd
DRC 457
Saint Louis, MO 63104
PI: Mee-Ngan F. Yap

Site 2: July 01, 2019-Dec 2019[A]
Northwestern University [A]
320 E. Superior St.
Searle 3-430
Chicago, IL 60611
PI: Mee-Ngan F. Yap

[A] Due to transfer of the institution, the remaining one-third of the proposed research will be performed at Northwestern University starting July 2019. We expect to have 3-5 weeks delay in research progress because the new laboratory will not be in full operation for at least 2 weeks.

[B] Affinity purification and sensitivity testing of the antibody will be continued at Northwestern University (Site 2).

[C] In the remaining ~6 months, we will focus on phage display screening of m^6A-ribosome binders (Aim 3)

Specific Aim 1	Timeline	Site 1	Site 2
Development of antibody-oligo conjugates	Months		
Oligo synthesis and antibody conjugation	1-3	Dr. Yap	-
Determine specificity by affinity purification of ribosome	3-6	Dr. Yap	-
Establish immunoassays to detect m^6A-ribosome	3-6	Dr. Yap	-
Milestone(s) Achieved	12	Dr. Yap	-
Specific Aim 2			
Antibody production with KLH-m^6A-RNA conjugate			
Antigen preparation: conjugation of KLH to m^6A-RNA	Ongoing work prior to DoD funding in July 2018	Dr. Yap	-
Raise antibody in rabbits	Ongoing work prior to DoD funding in July 2018. The PI's startup fund was used to pay for antibody production	Josman, LLC	-
Testing antibody titer and specificity, purify antibody	1-18[B]	Dr. Yap	Dr. Yap

Establish immunoassays to detect m^6A-ribosome in single-species and mixed-species bacterial cultures	6-18[B]	Dr. Yap	Dr. Yap
Local IRB/IACUC Approval	N/A, See comments above	-	-
Milestone(s) Achieved:	12-18	Dr. Yap	
Specific Aim 3			
Aim 3. Phage display and peptide enrichment			
Library screening	13-18[C]	-	Dr. Yap
Selection, purification, and enrichment	13-18 [C]	-	Dr. Yap
SELEX	13-18 [C]	-	Dr. Yap
Milestone(s) Achieved:	18	-	Dr. Yap

If human subjects are involved in the proposed study, please provide the projected quarterly enrollment in the following table**. (Not applicable)**

	Year 1				**Year 2**				**Year 3**
Target Enrollment (per quarter)	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**
Site 1	14	14	42	42	42	42	28		
Site 2		14	14	42	42	42	42	14	
Site 3			14	42	42	42	42	14	2
Target Enrollment (cumulative)	**14**	**42**	**112**	**238**	**364**	**490**	**602**	**630**	**632**

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