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14. ABSTRACT: This project aligns directly with the current FY17 PRMRP topic area antimicrobial resistance. The research focuses on development and preclinical investigation of bacteriophages as novel antimicrobial agents for treatment and prevention of infections caused by multidrug resistant bacteria (MDRB). MDRB cause life-threating infections involving wounds, penetrating abdominal injuries, hospital acquired pneumonia, and sepsis. These infections are important global public health diseases that impact severely on our wounded war-fighters, veterans, and their hospitalized family members. The resulting shortage of safe and effective antibiotics poses a great challenge to the care of wounded military personnel, as well as civilians. Our objective in this study will be to develop a transformational strategy beyond the next logical steps of using bacteriophages for treatment and prevention of MDRB infections. Bacteriophages are emerging experimentally and clinically as important antimicrobial agents in treatment of refractory and potentially lethal MDRB infections Recent developments in the field have suggested that in addition to being potent antibacterial agents, additional efficacy can be obtained by utilization of phages that are able to modulate the severity of bacterial infections by selecting for bacterial mutants that are reduced in virulence or that have increased susceptibility to antibiotics. Among the most prevalent and lethal MDRB are Staphylococcus aureus (MRSA), carbapenemase producing Klebsiella pneumoniae (KPC), Acinetobacter baumannii, and Pseudomonas aeruginosa. We will study KPC as a model MDRB.

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February 28 2020

AnnMarie Gersch, Ph.D.
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RE: W81XWH-18-1-0724

"Innovative Strategies for Treating and Preventing Combat-Related Multidrug Resistant Bacterial Infections." Department of Defense, Congressionally Directed medical Research Program (CDMRP) Peer Reviewed Medical Research Program (PRMRP) Focused Grant.

Dear Dr. Gersch,

Enclosed is an interim Annual Report of our study, Innovative Strategies for Treating and Preventing Combat-Related Multidrug Resistant Bacterial Infections.

MIC Data

To assess possible phenotypes representing changes in antimicrobial susceptibility among the bacterial strains collected *in vivo* from the mouse model or generated *in vitro* by serial bacteriophage passaging we conducted several sets of minimum inhibitory concentration (MIC) assays. The first set of assays was conducted with 10 strains and represents one replicate. Commercial MIC plates from Sensititre were used, and the plate layout as given by Thermo-Fisher can be found here. http://www.trekds.com/products/sensititre/files/ESBL.pdf

The MIC plates used for this experiment were designed specifically for diagnostic use on extended beta-lactamase producing bacterial strains and contained concentrations of many antibiotics used to treat partially antibiotic resistant bacterial strains. For all MIC plates, the inoculum of bacteria was standardized to between $1x10^5$ and $1x10^6$ CFU using a 0.5 McFarland turbidity standard and immediate serial dilution. In this first set of MICs, the ESBL plate comes

lyophilized. Following the dilution to $1x10^6$ CFU/mL 100uL of each the bacterial strain of interest was added to each well of the ESBL plate, the plate was sealed, and incubated at 37C for 18hours before being read the next day. Each well was given either a positive or negative score for growth, defined as the well having any growth being positive. From these positive/negative scores, the actual MIC was generated with the lowest concentration of antibiotic that inhibited bacterial growth being considered the MIC for that strain/antibiotic combination.

We found that there were some minor changes in MIC for Ceftazidime and Cefotaxime when comparing the results of the WT strain and the phage resistant in vitro/ in vivo bacterial mutants. The results have been summarized in Table 1 below.

The results, however, were challenging to interpret because the strains used in this study were so highly antibiotic resistant that they frequently exceeded the highest MIC measured on the commercial ESBL plate. Some strains also had an intermediate phenotype at the highest concentrations but were still considered positive growth for this assay. Without having higher concentrations of antibiotic present on the plate, we could not determine if there was any change in MIC for some of the antibiotics tested.

Based on this knowledge, we decided to test the bacterial strains on new MIC plates made in house which contained antibiotics at higher concentrations. The plate was designed as shown in Table 2 below. Six antibiotics were selected based on current clinical applications, and each was tested either alone or with an appropriate beta-lactamase inhibitor at concentrations much higher than normal present on commercial MIC plates. Antibiotics included, Cefotaxime, Ceftazidime, Meropenem, Aztreonam, Imipenem, and Piperacillin. Beta-lactamase inhibitors included, Avibactam, Relebactam, and Tazobactam. The plate design is also shown with the 2019 CLSI guidelines for antibiotic susceptibility marked in green (susceptible), yellow (intermediate), and red (resistant) for context. All antibiotics were mixed in Mueller-Hinton broth and diluted according to the plate map, all numbers represent final ug/mL concentrations in each well. The beta-lactamase inhibitors were added at a fixed rate with a final concentration of 4ug/mL per well. Bacterial samples were again adjusted using a 0.5 McFarland Standard and dilution and a concentration of $5x10^5$ was achieved in each well. Final volumes in wells was 200uL. The plates were covered, and incubated at 37C for 18h before being read as previously described. These assays were conducted in triplicate and the results were averaged.

In Table 3 below, the results of 4 of the antibiotics are shown. These 4 MIC results were selected because they showed differences between strains. For all the remaining antibiotics and antibiotic/beta lactamase inhibitor combinations, there were no differences between strains. All strains were completely resistant to Piperacillin, Piperacillin/Tazobactam, and

Aztreonam. Whenever, an antibiotic was paired with Avibactam or Relebactam, no growth was observed at the tested concentrations.

The exception to these data was a control strain of *K.pneumoniae* (1776c), which was susceptible to all antibiotics tested at these concentrations as was expected. From these data, it is possible to see that there are changes in the antibiotic susceptibility of the bacteriophage resistant mutants. These changes, however, appear to be strain dependent and antibiotic dependent and were observed to both increase and decrease antibiotic susceptibility.

Phage & CAZ/AVI Data

To better understand the protective phenotype seen in the in vivo experiments where mice were best protected by a treatment of both bacteriophage and Ceftazidime/Avibactam (CAZ/AVI) we attempted to replicate this synergy in vitro with a plate assay. This assay was designed using the effective concentrations observed in the MIC experiments, as well as previous bacteriophage growth inhibition assays. This “checkerboard” assay represents different combinations of bacteriophage and CAZ/AVI and compares how each combination inhibits growth of a given bacterial strain following an 18 hour incubation. Combinations included, the bacteriophage at 10^7 to 10^3 PFU or No Phage, and CAZ/AVI from 16 to 0.025ug/mL or No Antibiotic. Bacterial cultures were added at $5x10^5$ CFU final concentration as described previously. Final well volumes were 200ul and plates were covered, and incubated 18h at 37C before results were read using a plate reader measuring absorbance at 550nm. Bacterial strains tested were, 39427 WT, 39427 Pharr Resistant Mutant, 39427 EI resistant Mutant, and 39427 JR Resistant Mutant. These were all in vitro generated bacteriophage resistant bacterial mutants. The chromosomal and plasmid sequence mapping of the resistant mutants are depicted in Figures 1-5 (attached in Email).

Results shown in Table 4 represent 3 averaged replicates. In general for all tested strains, higher combinations of antibiotics and phage were more successful at inhibiting growth than either the corresponding antibiotic or phage concentration alone. These results indicate that there is benefit to treating with bacteriophage and antibiotics rather than either group alone.

These data provide important observations that there is *in vitro* synergy between beta-lactam/beta-lactamase combinations and bacteriophage that requires study *in vivo* and that may have important clinical implications.

Our plans therefore are to replicate the synergy studies in vitro, to conduct these studies testing the hypothesis of synergy in vivo and to further genetically characterize the resistant mutants to understand the bacteriophage-induced downward shift in MICs.

Respectfully submitted,

Sincerely,

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Table 1: Minimum Inhibitory Concentration Assays of using Commercial ESBL Plates

Antibiotics	Experimental Strain 39427 WT	39427 Pharr(R)in Vitro	39427 Pharr(R) JR (R) in vitro	39427 Pharr (R) EI (R) in vitro	OB 6/1/17 Experimental Sample Bacteria only
AXO- Ceftriaxone	>128	>128	>128	>128	>128
CEP- Cephalothin	>16	>16	>16	>16	>16
FOT- Cefotaxime	>64	>64	>64	>64	>64
F/C- Cefotaxime/ clavulanic acid	<64/4	<64/4	<32/4	>64/4	<64/4
TAZ- Ceftazidime	>128	>128	>=128 R	>128	>128
T/C- Cefazidime/ clavulanic acid	>128/4	>128/4	>128/4	>128/4	>128/4
IMI- Imipenem	>16	>16	>16	>16	>16
FEP- Cefepime	>16	>16	>16	>16	>16
POD- Cefpodoxime	>32	>32	>32	>32	>32
FOX- Cefoxitin	>64	>64	>64	>64	>64
P/T4- Piperacillin/ tazobactam	>64/4	>64/4	>64/4	>64/4	>64/4
MERO- Meropenem	>8	>8	>8	>8	>8
GEN- Gentamicin	<4	<4	<4	<4	<4
CIP- Ciprofloxacin	>2	>2	>2	>2	>2
AMP- Ampicillin	>16	>16	>16	>16	>16
FAZ- Cefazolin	>16	>16	>16	>16	>16
Antibiotics	1A 6/2/17 Experimental Bacteria + phage treatment	19-3C 6-30-17 Bacteria/ Phage / 80mg/kg CAZ AVI	20B 6/30/17 bacteria / phage / 40mg/kg CAZ/AVI	20-2C-bacteria / phage / 40mg/kg CAZ/AVI	Antibiotic Sensitive Control Strain 1776c
AXO- Ceftriaxone	>128	>128	>128	>128	<1
CEP- Cephalothin	>16	>16	>16	>16	<8
FOT- Cefotaxime	>64	>64	>64	>64	<.5
F/C- Cefotaxime/ clavulanic acid	>64/4	>64/4	>16/4	>64/4	<.25/4
TAZ- Ceftazidime	>128	>128	<128 R	>128	<.5
T/C- Cefazidime/ clavulanic acid	>128/4	>128/4	>64/4 R	>128/4	<.25/4
IMI- Imipenem	>16	>16	<8 R/I	>16	<.5
FEP- Cefepime	>16	>16	>16	>16	<1
POD- Cefpodoxime	>32	>32	>32	>32	<1
FOX- Cefoxitin	>64	>64	>64	>64	>64
P/T4- Piperacillin/ tazobactam	>64/4	>64/4	>64/4	>64/4	<4/4
MERO- Meropenem	>8	>8	>8	>8	<1
GEN- Gentamicin	<4	<4	<4	<4	<4
CIP- Ciprofloxacin	>2	>2	>2	>2	>2
AMP- Ampicillin	>16	>16	>16	>16	>16
FAZ- Cefazolin	>16	>16	>16	>16	>16
Blue= Intermediate Observed					
Yellow= Change Observed					

Table 2: Design of High Concentration MIC Plate for Testing Bacterial Mutant Antibiotic Susceptibility

2019 Walsh Model InVivo and InVitro K.pneu Samples						S	I	R				
<>	1	2	3	4	5	6	7	8	9	10	11	12
A	FOT16	F/C16	TAZ8	T/A8	MER8	M/A8	P8	P/T8	AZT2	A/A2	IMI2	I/R2
B	FOT32	F/C32	TAZ16	T/A16	MER16	M/A16	P16	P/T16	AZT4	A/A4	IMI4	I/R4
C	FOT64	F/C64	TAZ32	T/A32	MER32	M/A32	P32	P/T32	AZT8	A/A8	IMI8	I/R8
D	FOT128	F/C128	TAZ64	T/A64	MER64	M/A64	P64	P/T64	AZT16	A/A16	IMI16	I/R16
E	FOT256	F/C256	TAZ128	T/A128	MER128	M/A128	P128	P/T128	AZT32	A/A32	IMI32	I/R32
F	FOT512	F/C512	TAZ256	T/A256	MER256	M/A256	P256	P/T256	AZT64	A/A64	IMI64	I/R64
G	FOT1024	F/C1024	TAZ512	T/A512	MER512	M/A512	P512	P/T512	AZT128	A/A128	IMI128	I/R128
H	POS	POS	POS	NEG	NEG	NEG	P1024	P/T1024	AZT256	A/A256	IMI256	I/R256

CLSI 2019					
			S	I	R
FOT	Cefotaxime		≤ 1	2	≥ 4
F/C	Cefotaxime/ Avibactam				
TAZ	Ceftazidime		≤ 4	8	≥ 16
T/A	Ceftazidime/ Avibactam		≤ 8/4		≥ 16/4
MERO	Meropenem		≤ 1	2	≥ 4
M/A	Meropenem/ Avibactam		≤ 4/4		≥ 16/4
AZT	Aztreonam		≤ 4	8	≥ 16
A/A	Aztreonam/ Avibactam				
IMI	Imipenem		≤ 1	2	≥ 4
I/R	Imipenem/ Relebactam				
P	Piperacillin		≤ 16	32–64	≥ 128
P/T	Piperacillin/ Tazobactam		≤ 16/4	32/4–64/4	≥ 128/4
NEG	Media				
POS	Positive Control				

Table 3:Selected MIC Results for High Concentration MIC Assay

Antibiotic	Experimental Strain 39427 WT	39427 Pharr(R)in Vitro	39427 Pharr(R) JR (R) in vitro	1A Experimental Bacteria + phage treatment	1B Experimental Bacteria + phage treatment
Cefotaxime	256	>1024	128	1024	1024
Ceftazidime	512	512	128	>512	512
Meropenem	64	128	128	256	64
Imipenem	64	128	128	128	64

Antibiotic	19-3C Bacteria/ Phage / 80mg/kg CAZ AVI	19-4C Bacteria/ Phage / 80mg/kg CAZ AVI	20-2C-bacteria / phage / 40mg/kg CAZ/AVI	20-4C-bacteria / phage / 40mg/kg CAZ/AVI
Cefotaxime	128	128	512	1024
Ceftazidime	512	512	256	>512
Meropenem	64	64	64	256
Imipenem	64	64	128	128

Table 4: Bacteriophage & CAZ/AVI Checkerboards

	39427 WT					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.24	0.08	0.05	0.04	0.02	0.00
8ug Caz 2ug Avi	0.54	0.34	0.16	0.17	0.07	0.05
4ug Caz 1ug Avi	0.57	0.34	0.30	0.16	0.14	0.04
2ug Caz 0.5ug Avi	0.59	0.35	0.28	0.30	0.07	0.06
1ug Caz 0.25ug Avi	0.62	0.38	0.24	0.19	0.08	0.12
0.5ug Caz 0.125ug Avi	0.59	0.28	0.29	0.14	0.12	0.07
.025ug Caz 0.0625ug Avi	0.53	0.34	0.21	0.20	0.11	0.07
No Antibiotic	0.61	0.28	0.13	0.15	0.12	0.08
	39427 Pharr Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.22	0.09	0.06	0.04	0.02	0.00
8ug Caz 2ug Avi	0.53	0.36	0.14	0.14	0.08	0.05
4ug Caz 1ug Avi	0.45	0.26	0.24	0.12	0.06	0.03
2ug Caz 0.5ug Avi	0.59	0.36	0.26	0.28	0.06	0.06
1ug Caz 0.25ug Avi	0.63	0.39	0.26	0.19	0.11	0.10
0.5ug Caz 0.125ug Avi	0.63	0.32	0.31	0.20	0.08	0.07
.025ug Caz 0.0625ug Avi	0.63	0.35	0.24	0.22	0.09	0.09
No Antibiotic	0.64	0.30	0.17	0.17	0.12	0.07
	39427 EI Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.23	0.11	0.10	0.08	0.04	0.00
8ug Caz 2ug Avi	0.61	0.49	0.31	0.34	0.34	0.33
4ug Caz 1ug Avi	0.50	0.39	0.24	0.25	0.24	0.23
2ug Caz 0.5ug Avi	0.64	0.39	0.33	0.35	0.34	0.36
1ug Caz 0.25ug Avi	0.68	0.39	0.32	0.35	0.35	0.36
0.5ug Caz 0.125ug Avi	0.68	0.37	0.32	0.35	0.34	0.34
.025ug Caz 0.0625ug Avi	0.68	0.44	0.34	0.37	0.35	0.36
No Antibiotic	0.56	0.35	0.34	0.37	0.35	0.35
	39427 JR Resistant					
	No Phage	10^3	10^4	10^5	10^6	10^7
16ug Caz 4ug Avi	0.11	0.09	0.10	0.07	0.05	0.00
8ug Caz 2ug Avi	0.47	0.44	0.42	0.40	0.41	0.41
4ug Caz 1ug Avi	0.36	0.35	0.34	0.33	0.31	0.31
2ug Caz 0.5ug Avi	0.49	0.49	0.46	0.44	0.43	0.44

1ug Caz 0.25ug Avi	0.50	0.49	0.46	0.46	0.46	0.48
0.5ug Caz 0.125ug Avi	0.51	0.49	0.47	0.46	0.45	0.47
.025ug Caz 0.0625ug Avi	0.54	0.50	0.47	0.46	0.45	0.45
No Antibiotic	0.51	0.49	0.46	0.46	0.46	0.46

39427 Bacterial Genome & Proposed Mutations

Following extensive genomic assembly, we currently hypothesize that the 39427 bacterial genome is a circular chromosome of 5,426kb in length with 3 plasmids 168kb, 57kb, and 89kb in length. This is being confirmed by further sequencing for closure. Using this sequence, and the sequence of some of our bacteriophage resistant bacterial mutants, we have identified probable causative mutations for protection against some of our bacteriophage. These genes are currently being complemented to confirm they are responsible for the phage resistant phenotype. Additionally, this closed genome is allowing us to annotate various virulence factors, which may affect by the phage treatment. An analysis is forthcoming.

Bacterial Strain	Type of Mutation	Gene
39427 (Pharr-R)	+1 frameshift, L29	undecaprenyl-phosphate galactose phosphotransferase *wbaP*
39427 (Soft-R)	+1 frameshift, L29	undecaprenyl-phosphate galactose phosphotransferase *wbaP*
39427 (Pharr-R) (Spivey-R1)	-1 frameshift, R316	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R1)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions
39427 (Pharr-R) (Spivey-R2)	11 bp deletion starting in P169	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R2)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions
39427 (Pharr-R) (Spivey-R3)	+1 frameshift, Y106	Ferrichrome transporter *fhuA*
39427 (Pharr-R) (Spivey-R3)	86,758 bp deletion	Region contains CPS and O-Antigen coding regions