

A Broad-Spectrum Oral Bacteriophage Cocktail for Prevention of Gastrointestinal Infections in Austere and Deployed Environments

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Gastrointestinal (GI) infections remain a leading cause of morbidity among deployed U.S. forces, significantly impacting mission readiness and operational effectiveness. The increasing prevalence of antibiotic-resistant enteric pathogens further limits treatment options in austere environments. Bacteriophages (phages), viruses that selectively infect and lyse bacteria, offer a targeted, microbiome-sparing alternative to antibiotics. Lytic phages are recognized as safe (GRAS) and suitable for oral delivery. However, developing stable, broad-spectrum phage cocktails for deployment remains a critical challenge. This comprehensive study evaluated Theralytix™ Enteric Bacteriophage, a multi-phage formulation targeting key gastrointestinal pathogens. A standardized 12-phage cocktail targeting *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. was produced, purified, and combined to achieve consistent titers and broad host range. Phage DNA was extracted and sequenced. Antimicrobial efficacy was assessed using spot and zone-of-inhibition assays against clinical and antibiotic-resistant isolates. Cytotoxicity was evaluated in L929 fibroblast cells using a modified direct-contact approach in accordance with ISO 10993-5. Phage potency (PFU/mL), sterility, and endotoxin levels were quantified. Stability was assessed under physiological conditions, real-time storage (25°C and 2–8°C), and extreme environmental conditions (–20°C to 70°C, including UV exposure) to simulate operational deployment. The 12-phage cocktail demonstrated robust, broad-spectrum lytic activity against all tested isolates, including antibiotic-resistant strains. Genomic analysis confirmed that all phages are strictly lytic. Cytotoxicity testing showed no significant adverse effects on mammalian cells, and endotoxin levels were within acceptable limits. Stability studies confirmed maintenance of phage viability, with no significant reduction in titer or potency across physiological, storage, and extreme environmental conditions, including thermal and freeze–thaw stress, supporting suitability for field deployment. The Bacteriophage cocktail represents a promising oral, over the counter (OTC) countermeasure for prevention and control of gastrointestinal infections in military and civilian populations. Its demonstrated efficacy, safety, and stability support its potential as a deployable solution in resource-limited and austere environments.

Clinical and Military Relevance

- ❑ Gastrointestinal (GI) infections remain a leading cause of morbidity among deployed U.S. forces, significantly impacting mission readiness and operational effectiveness.
- ❑ Bacteriophages selectively lyse bacteria and offer a promising alternative to antibiotics.
- ❑ Phage cocktails show strong potential for treating GI infections.
- ❑ This work was conducted as part of an **U.S. Air Force SBIR Phase II** contract for development of bacteriophage therapeutics against Diarrhea, Dysentery and Urinary Tract Infections.

Objectives

- ❑ To prepare, test, and analyze data for successful production of *E. coli*, *Salmonella*, and *Shigella* phages.
- ❑ To prepare a standardized 12-phage cocktail by combining individual phages targeting specific bacterial strains in defined ratios to achieve broad-spectrum activity and consistent titer.



E. coli Bacteriophage



Phage Top Agar Overlay

Preparation and Evaluation of Phage Cocktails

- ❑ A standardized 12-phage cocktail targeting *E. coli*, *Salmonella* spp., and *Shigella* spp. was produced to achieve consistent titers and broad host range.
- ❑ Phage DNA was extracted and sequenced.
- ❑ Cytotoxicity was evaluated in L929 fibroblast cells using a modified direct-contact approach in accordance with ISO 10993-5.
- ❑ Phage potency (PFU/mL), sterility, and endotoxin levels were determined.
- ❑ Stability was evaluated under physiological, real-time (25°C, 2–8°C), and extreme environmental conditions (–20°C to 70°C, UV exposure) to simulate deployment.

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Zone of Inhibition Testing Results

- ❑ Antimicrobial efficacy was assessed using spot and zone-of-inhibition assays against clinical and antibiotic-resistant isolates.
- ❑ Bacterial lawns were prepared by plating 200 µL of 1.5 × 10⁷ CFU/mL of bacterial culture and incubating at 37°C.
- ❑ Phage cocktail (10 µL) was spotted onto plates and incubated for 24-48 hours.
- ❑ Killing efficacy was determined by measuring zones of inhibition (ZOI).

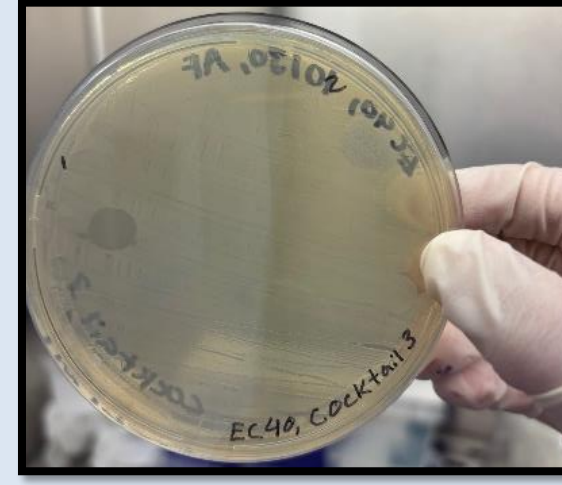
Observation : Y: Clear zone of inhibition (ZOI); N: No zone of inhibition; Plaque size measurements are in millimeters (mm)



E. coli #3



E. coli #6



E. coli #40



Salmonella enterica NR-170



Shigella sonnei #1



Shigella flexneri #31

Individual Phage Preparation and DNA Extraction

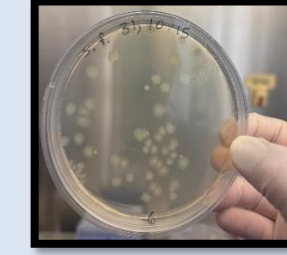
Individual Phage Preparation Data					Individual Phage DNA Extraction Concentration Results		
Individual Phage	Host Bacteria	Phage Titer PFU/ml		Purified Phage (0.22 µm filter) Media: Sterility Results	Individual Phage	A260/A280	DNA Conc (ng/µL)
		Before Amplification	After Amplification				
Phage #1	<i>E. coli</i> #6	4.60 x 10 ⁸ PFU/ml	4.60 x 10 ⁸ PFU/ml	TSA: Pass	Phage #1	1.68	13
Phage #2	<i>E. coli</i> #40	7.00 x 10 ⁸ PFU/ml	1.03 x 10 ⁸ PFU/ml	TSA: Pass	Phage #2	1.37	24.8
Phage #5	<i>E. coli</i> #3	1.58 x 10 ⁸ PFU/ml	4.60 x 10 ⁸ PFU/ml	TSA: Pass	Phage #5	1.7	58
Phage #7	<i>Salmonella</i> NR-170	2.40 x 10 ⁸ PFU/ml	1.01 x 10 ¹⁰ PFU/ml	TSA: Pass	Phage #7	1.83	114.7
Phage #8	<i>Salmonella</i> NR-170	5.00 x 10 ⁶ PFU/ml	2.32 x 10 ¹⁰ PFU/ml	TSA: Pass	Phage #8	1.84	439.8
Phage #9	<i>Salmonella</i> NR-170	7.80 x 10 ³ PFU/ml	2.18 x 10 ¹⁰ PFU/ml	TSA: Pass	Phage #9	1.8	122.8
Phage #13	<i>Shigella sonnei</i> #1	6.40 x 10 ⁷ PFU/ml	5.00 x 10 ¹⁰ PFU/ml	TSA: Pass	Phage #13	1.86	78.9
Phage #14	<i>Shigella flexneri</i> #31	5.40 x 10 ⁶ PFU/ml	5.00 x 10 ¹⁰ PFU/ml	TSA: Pass	Phage #14	1.68	52.7



E. coli #6 Host Bacteria



Salmonella Strain NR-170 Host Bacteria



Shigella flexneri Strain #31 Host Bacteria

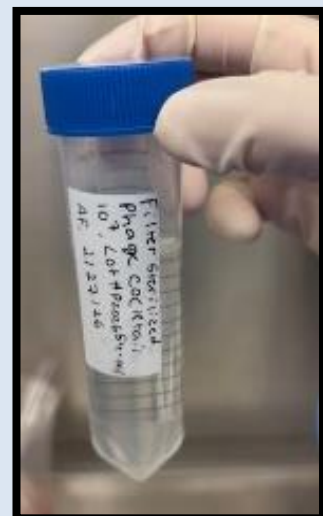


Phage DNA Isolation Kit

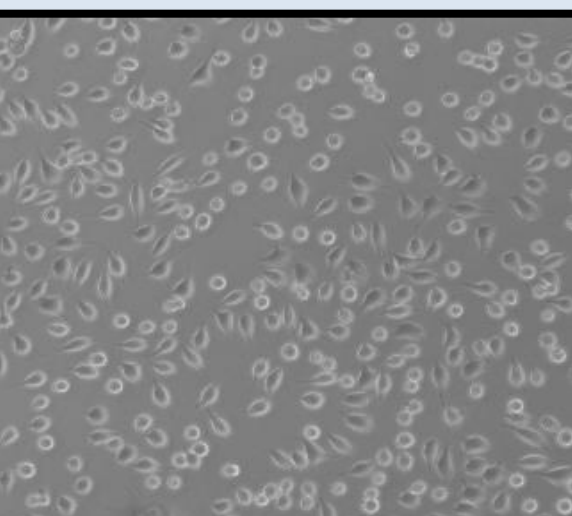


Nanodrop

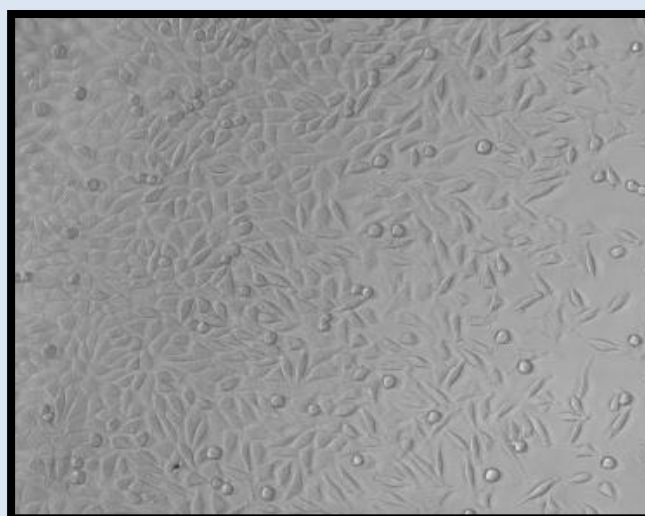
Phage Cocktail Cytotoxicity Testing



Phage Cocktail containing 12 Individual Phages



Mouse Fibroblast (L929 Clone) Cell Line With DMEM Media Only



Mouse Fibroblast (L929 Clone) Cell Line treated with Phage Cocktail

Phage Cocktail Stability Testing Results

Results of the 12 Individual Phages in the Liquid Cocktail Based on Host Bacteria
Real Time Stability Storage: 25 °C ± 2 °C and 60% RH ± 5%

Host Bacteria	0-Month Titer (PFU/mL)	1-Month Titer (PFU/mL)	2-Month Titer (PFU/mL)	3-Month Titer (PFU/mL)
<i>E. coli</i> Strain # 6	1.26 X 10 ⁸	1.00 X 10 ⁸	7.00 X 10 ⁷	6.70 X 10 ⁷
<i>E. coli</i> Strain # 40	1.20 X 10 ⁸	4.60 X 10 ⁷	3.70 X 10 ⁷	3.20 X 10 ⁷
<i>E. coli</i> Strain # 3	1.04 X 10 ⁸	1.01 X 10 ⁸	6.10 X 10 ⁷	4.60 X 10 ⁷
<i>E. coli</i> Strain # 31	2.21 X 10 ⁸	1.56 X 10 ⁸	1.24 X 10 ⁸	1.18 X 10 ⁸
<i>E. coli</i> Strain # 54	2.20 X 10 ⁸	1.74 X 10 ⁸	1.07 X 10 ⁸	9.80 X 10 ⁷
<i>Salmonella</i> NR-170	2.30 X 10 ⁷	1.68 X 10 ⁷	3.10 X 10 ⁷	5.00 X 10 ⁷
<i>Shigella sonnei</i> #0871	7.30 X 10 ⁸	3.18 X 10 ⁸	8.80 X 10 ⁷	7.40 X 10 ⁷
<i>Shigella flexneri</i> #31	7.10 X 10 ⁸	1.18 X 10 ⁸	9.80 X 10 ⁷	7.20 X 10 ⁷

Phage Cocktail Liquid Sample Batch
Extreme Condition (40 °C ± 2 °C and 75% RH ± 5%)

Host Bacteria	Time 0 Titer (PFU/mL)	Time Week 1 Titer (PFU/mL)	Time Week 2 Titer (PFU/mL)	Time Week 4 Titer (PFU/mL)
<i>E. coli</i> Strain # 6	1.26 X 10 ⁸	4.30 X 10 ⁷	5.50 X 10 ⁶	3.50 X 10 ⁷
<i>E. coli</i> Strain # 40	1.20 X 10 ⁸	1.53 X 10 ⁷	1.18 X 10 ⁷	8.60 X 10 ⁶
<i>E. coli</i> Strain # 3	1.04 X 10 ⁸	1.58 X 10 ⁷	1.48 X 10 ⁷	1.07 X 10 ⁷
<i>E. coli</i> Strain # 31	2.21 X 10 ⁸	9.60 X 10 ⁷	9.70 X 10 ⁷	7.30 X 10 ⁷
<i>E. coli</i> Strain # 54	2.20 X 10 ⁸	8.40 X 10 ⁷	6.50 X 10 ⁷	5.20 X 10 ⁷
<i>Salmonella</i> NR-170	2.30 X 10 ⁷	1.73 X 10 ⁷	7.70 X 10 ⁶	5.90 X 10 ⁶
<i>Shigella sonnei</i> #0871	7.30 X 10 ⁸	1.01 X 10 ⁸	6.20 X 10 ⁷	5.30 X 10 ⁷
<i>Shigella flexneri</i> #31	7.10 X 10 ⁸	1.24 X 10 ⁸	5.90 X 10 ⁷	4.80 X 10 ⁷

Conclusions

- ❑ Stability studies demonstrated sustained phage viability and potency under physiological, storage, and extreme environmental conditions, supporting field deployment.
- ❑ The Bacteriophage cocktail represents a promising oral, over the counter (OTC) countermeasure for prevention and control of gastrointestinal infections in military and civilian populations.
- ❑ Its demonstrated efficacy, safety, and stability support its potential as a deployable solution in resource-limited and austere environments.