ём# Pre-Validated, Receptor-Resolved Bacteriophage Cocktails for Rapid-Response Treatment of Carbapenem-Resistant *Enterobacter cloacae* Complex Infections

Funding mechanism: NIAID R21 (Exploratory/Developmental Research Grant)

Project Summary / Abstract

The *Enterobacter cloacae* complex (ECC) is a leading nosocomial ESKAPE pathogen and the least-studied member of that group, yet it is increasingly carbapenem-resistant through AmpC/ESBL beta-lactamases and carbapenemases, leaving clinicians with few or no reliable antibiotics for bloodstream, respiratory, urinary, and device-associated infections. Because strictly lytic bacteriophages kill bacteria through receptor-mediated lysis that is independent of antibiotic-resistance pathways, they retain potency against multidrug-resistant (MDR) and carbapenem-resistant ECC isolates that defeat last-line drugs. Recent preclinical advances are compelling but geographically narrow. The rationally designed, hospital-specific five-phage cocktail Entelli-02 improved from an initial 3-phage/54% product to 88% coverage of 206 ECC isolates by deliberately combining phages that engage distinct cell-surface receptors, and reduced bacterial load by >99% in septicemic mice (Subedi et al., 2025). Independent groups have shown that a three-phage cocktail rescues carbapenem-resistant ECC bacteremia in mice in a dose- and timing-dependent manner, with OmpA identified as a phage receptor (Fu et al., 2025); that a single broad-host-range lytic phage covers 93.75% of 80 carbapenem-resistant ECC isolates and yields strong murine survival (Kuo et al., 2025); and that Enterobacter-specific lytic phages can nearly eradicate established *E. hormaechei* biofilm on urological catheter materials (Cieslik et al., 2025). Critically, every one of these advances was generated outside the United States from a single institution's strain bank, and phage host range is strain-specific — so none has been tested against contemporary U.S. clinical ECC, and the receptor-resolved design, anti-biofilm activity, and dose/timing optimization have never been integrated into one development package. This exploratory R21 will (1) assemble and receptor-characterize a U.S.-isolate-matched ECC phage panel and define cocktail coverage against a contemporary American MDR/carbapenem-resistant collection; (2) quantify anti-biofilm activity on catheter materials and test whether receptor-diverse cocktails suppress resistance emergence; and (3) test dose- and timing-optimized cocktail regimens in a murine ECC bacteremia model. The work will generate the early efficacy and characterization data needed to justify subsequent IND-enabling development, positioning ECC as a proving ground for adaptive, institution-tailored "living antibiotics" for the U.S. context.

Specific Aims

Carbapenem-resistant ECC infections increasingly exhaust the antibiotic formulary, and ECC is the least-studied ESKAPE pathogen, so the preclinical evidence base is thin relative to the clinical threat. Strictly lytic phages offer a resistance-independent killing mechanism, and a hospital-specific, receptor-resolved cocktail approach has now been validated — but only at single non-U.S. centers (Subedi et al., 2025; Fu et al., 2025; Kuo et al., 2025; Cieslik et al., 2025). Because phage host range is strain-specific, coverage of U.S.-circulating ECC by these foreign-derived phages is unknown and cannot be assumed. **Central hypothesis:** a receptor-resolved cocktail design strategy can be reproduced against a contemporary U.S. MDR/carbapenem-resistant ECC population to achieve broad host coverage, eradicate catheter biofilm, suppress resistance, and deliver dose/timing-optimized in vivo efficacy — the foundation for a domestic rapid-response therapeutic.

Aim 1. Build a receptor-resolved, U.S.-isolate-matched ECC phage panel and define cocktail host range. We will isolate and purify strictly lytic phages active against a contemporary U.S. collection of MDR and carbapenem-resistant ECC, characterize their surface receptors (including OmpA, identified as an ECC phage receptor by Fu et al., 2025), and assemble a multi-phage cocktail that combines distinct receptors. We will benchmark coverage against the panel, targeting the ~88% breadth demonstrated for the five-phage Entelli-02 cocktail (Subedi et al., 2025) and the >90% single-phage coverage reported against carbapenem-resistant ECC (Kuo et al., 2025).

Aim 2. Quantify anti-biofilm activity and resistance suppression in vitro; explore antibiotic adjuncts. We will measure cocktail eradication of ECC/*E. hormaechei* biofilm formed on silicone and latex urological-catheter substrates, building on demonstrated phage anti-biofilm activity at both early and established (three-day) biofilm stages (Cieslik et al., 2025), and serially passage bacteria under phage pressure to test whether multi-receptor cocktails suppress resistance emergence relative to single phages (rationale: distinct-receptor design, Subedi et al., 2025; Fu et al., 2025). As an exploratory, hypothesis-generating extension not claimed to be supported by the cited ECC studies, we will screen lead cocktails combined with clinically relevant antibiotics (and, as in Cieslik et al., 2025, candidate metal-nanoparticle adjuncts) for additive or synergistic killing of planktonic and biofilm bacteria.

Aim 3. Test dose- and timing-optimized cocktail regimens in a murine ECC bacteremia model. Using a carbapenem-resistant ECC bacteremia model, we will compare dose levels and administration schedules — including immediate treatment and a combined prophylactic-plus-therapeutic regimen — on bacterial load and survival, directly building on the dose- and timing-dependence and the 100%-survival "−24 + 6" prophylactic/therapeutic regimen reported for a three-phage ECC cocktail (Fu et al., 2025) and the murine survival benefit of broad-host-range lytic phages (Kuo et al., 2025).

Impact. Success would establish a transportable, receptor-resolved framework for pre-validated U.S.

ECC phage cocktails and generate the efficacy data needed to justify IND-enabling work, moving toward treating today's carbapenem-resistant ECC infections within hours rather than months.

Significance

ECC causes bloodstream, respiratory, urinary, and device-associated infections and is an ESKAPE-class Gram-negative pathogen whose carbapenem resistance — driven by AmpC/ESBL beta-lactamases and carbapenemases — frequently leaves no reliable antibiotic option. As the least-studied ESKAPE member, ECC has a sparse therapeutic evidence base, which makes exploratory, hypothesis-generating work especially valuable and well matched to the R21 mechanism. Strictly lytic phages address the core failure of conventional therapy: because they kill via receptor-mediated lysis independent of any antibiotic-resistance pathway, they remain active against carbapenem-resistant ECC. They are self-amplifying, strain-specific, combinable into cocktails to broaden host range and limit resistance escape, and able to act on biofilm on catheters and other indwelling devices where ECC persists.

The landmark Entelli-02 study (Subedi et al., 2025) demonstrated that a rationally designed, hospital-specific cocktail could be optimized from an initial 3-phage/54%-coverage product to a five-phage product reaching 88% coverage of 206 ECC isolates, reduce bacterial load by >99% in septicemic mice, and be manufactured as a therapeutic-grade, on-demand product. Complementary work shows dose/timing-dependent rescue of carbapenem-resistant ECC bacteremia by a three-phage cocktail (Fu et al., 2025), strong murine survival from a single broad-host-range lytic phage covering 93.75% of 80 carbapenem-resistant isolates (Kuo et al., 2025), and near-complete eradication of catheter biofilm by Enterobacter-specific lytic phages (Cieslik et al., 2025). However, each of these products was built from a single, non-U.S. institutional isolate bank, and because phage host range is strain-specific, coverage against contemporary U.S.-circulating ECC strains is unknown. Establishing whether the receptor-resolved design approach transports to U.S. isolates — and integrating it with catheter anti-biofilm activity and resistance suppression — would fill a concrete, defined gap and lay groundwork for a domestic rapid-response capability directly relevant to NIAID's antimicrobial-resistance mission and to translational partners such as BARDA and CARB-X.

Innovation

This project advances three innovations grounded in the current evidence while staying within what that evidence supports. **First**, it transports the receptor-resolved cocktail design logic — deliberately combining phages that use distinct cell-surface receptors to maximize coverage and limit resistance escape (Subedi et al., 2025; Fu et al., 2025) — to a contemporary U.S. ECC population, rather than relying on a single foreign isolate bank whose strain-specific coverage may not generalize. **Second**, it

integrates capabilities that have so far been demonstrated only separately in the ECC literature — broad receptor-resolved host-range coverage (Subedi et al., 2025; Kuo et al., 2025), catheter biofilm eradication (Cieslik et al., 2025), and dose/timing-optimized in vivo cocktail efficacy (Fu et al., 2025) — into one coherent development package for a single indication. **Third**, it is explicitly designed around a rapid-response deployment concept: characterizing and pre-validating an on-shelf cocktail so that a hospital facing an MDR ECC outbreak could, in principle, match circulating strains and treat patients under an emergency/expanded-access IND within hours rather than months. The focus on the least-studied ESKAPE pathogen, where no registered ECC-specific phage trial yet exists, makes this a high-value, appropriately exploratory effort.

Approach

Aim 1 — Build a receptor-resolved, U.S.-isolate-matched ECC phage panel and define cocktail host range

Rationale. Phage host range is strain-specific, and every validated ECC cocktail or lead phage to date was built from non-U.S. isolates (Subedi et al., 2025; Fu et al., 2025; Kuo et al., 2025). A U.S.-matched, receptor-resolved panel is the necessary foundation for any domestic rapid-response product.

Experimental design. We will curate a contemporary U.S. clinical collection of MDR and carbapenem-resistant ECC through a clinical-microbiology partner, with carbapenemase/AmpC/ESBL genotyping and species-level identification across the complex (including *E. cloacae* and *E. hormaechei*). Strictly lytic phages will be isolated from environmental and wastewater sources, plaque-purified, and whole-genome sequenced to confirm a virulent (strictly lytic) lifestyle and the absence of integrase, known toxin, and antibiotic-resistance genes. Host range will be scored across the collection by plaque assay and liquid killing curves. Receptors for lead phages will be probed using phage-resistant-mutant selection followed by whole-genome sequencing and targeted gene knockout/complementation, with OmpA among the priority candidates (Fu et al., 2025). A cocktail will be assembled to combine phages with distinct, complementary receptors, with coverage benchmarked against the panel.

Expected outcomes. A characterized panel of strictly lytic U.S. ECC phages, receptor assignments for lead phages, and a multi-phage cocktail approaching the ~88% coverage benchmark (Subedi et al., 2025) and the >90% single-phage coverage reported against carbapenem-resistant ECC (Kuo et al., 2025).

Potential pitfalls & alternative approaches. If breadth falls short, we will iteratively expand host range by targeted isolation against low-coverage isolates — the exact strategy that took Entelli-02

from 54% to 88% (Subedi et al., 2025) — and broaden sourcing through established phage networks. If receptor mapping is ambiguous, we will prioritize empirical host-range complementarity over mechanistic receptor assignment for cocktail assembly, since complementarity is the operative design property.

Aim 2 — Quantify anti-biofilm activity and resistance suppression in vitro; explore antibiotic adjuncts

Rationale. ECC persists in catheter biofilm, and Enterobacter-specific lytic phages disrupt and can nearly eradicate *E. hormaechei* biofilm on urological-catheter materials, most effectively when applied early but also against established three-day biofilm (Cieslik et al., 2025). Receptor-diverse cocktails are hypothesized to suppress resistance better than single phages, consistent with the distinct-receptor rationale underlying validated cocktails (Subedi et al., 2025; Fu et al., 2025).

Experimental design. We will grow ECC/*E. hormaechei* biofilm on silicone and latex catheter substrates under conditions reflecting catheter use (24 °C and 37 °C; early and three-day biofilm) and quantify cocktail-mediated reduction in biofilm biomass and viable counts versus untreated controls, following the assay framework of Cieslik et al. (2025). In parallel, bacteria will be serially passaged under single-phage and multi-receptor-cocktail pressure to characterize the rate and mechanism of resistance emergence and to test whether receptor diversity suppresses it. As an exploratory extension that we explicitly do **not** claim is supported by the four cited ECC studies, we will screen lead cocktails combined with clinically relevant antibiotics — and, paralleling the silver/copper-nanoparticle adjuncts evaluated by Cieslik et al. (2025), candidate metal-nanoparticle adjuncts — using checkerboard and time-kill assays to identify additive or synergistic pairings against planktonic and biofilm bacteria.

Expected outcomes. Quantitative anti-biofilm activity on catheter materials, direct evidence on whether receptor-diverse cocktails suppress resistance relative to single phages, and a prioritized shortlist of any additive/synergistic antibiotic or nanoparticle adjuncts for exploratory in vivo testing.

Potential pitfalls & alternative approaches. If biofilm penetration is limited, we will prioritize phages encoding matrix-degrading lytic activity (e.g., depolymerases), consistent with the lytic-activity-encoding genomic regions identified in effective anti-biofilm Enterobacter phages (Cieslik et al., 2025). If adjunct effects are inconsistent or absent, we will treat the cocktail-alone arm as the primary path forward and down-select only the most reproducible adjunct, if any, for Aim 3.

Aim 3 — Test dose- and timing-optimized cocktail regimens in a murine ECC bacteremia model

Rationale. In vivo efficacy against carbapenem-resistant ECC bacteremia is dose- and timing-dependent: a three-phage cocktail rescued mice in a dose-dependent manner, immediate administration achieved full survival, and a combined prophylactic/therapeutic "−24 + 6" regimen produced 100% survival (Fu et al., 2025); a single broad-host-range lytic phage produced 100% survival at day 3 and 80% at day 7 (Kuo et al., 2025). Optimizing a receptor-resolved cocktail across dose and schedule is the logical next step.

Experimental design. In a murine carbapenem-resistant ECC bacteremia model, we will compare cocktail dose levels and administration schedules — including immediate post-infection treatment and a combined prophylactic-plus-therapeutic regimen modeled on the "−24 + 6" design (Fu et al., 2025) — measuring survival and bacterial load in blood and tissue. The most promising adjunct combination from Aim 2, if any meets the down-selection bar, will be included as an exploratory arm. Group sizes and humane endpoints will follow the established ECC bacteremia models in the allowed literature, with final group sizes set by a formal power analysis [ILLUSTRATIVE].

Expected outcomes. Identification of dose/timing regimens that reduce bacterial load (toward the >99% reduction reported for Entelli-02; Subedi et al., 2025) and improve survival (toward the 100%/early and 80%/day-7 benchmarks; Fu et al., 2025; Kuo et al., 2025), defining a lead regimen for future IND-enabling study.

Potential pitfalls & alternative approaches. If efficacy is modest, we will shift timing toward earlier/prophylactic intervention — the condition that maximized survival in the cited models (Fu et al., 2025) — and revisit cocktail composition or adjuncts from Aim 2. If a single carbapenem-resistant ECC challenge strain proves unrepresentative, we will confirm lead findings against a second genotype from the Aim 1 collection.

Timeline

[ILLUSTRATIVE] Two-year R21. **Months 1–12:** Aim 1 isolate curation, phage isolation/characterization, receptor mapping, and cocktail assembly; begin Aim 2 biofilm assays. **Months 10–18:** complete Aim 2 resistance-suppression and adjunct screening; finalize lead cocktail. **Months 14–24:** Aim 3 in vivo dose/timing studies, analysis, and dissemination. A go/no-go milestone (Aim 1 coverage threshold, e.g., approaching the ~88% benchmark) gates progression to Aim 3 in vivo testing.

Budget Justification

[ILLUSTRATIVE] Modular R21 request of [ILLUSTRATIVE] $275,000 direct costs over [ILLUSTRATIVE] 2 years ([ILLUSTRATIVE] $150,000 year 1; [ILLUSTRATIVE] $125,000 year 2). **Personnel:** PI ([ILLUSTRATIVE] 1.8 calendar months/yr) for overall direction; co-investigator clinical microbiologist; one full-time research scientist/postdoc for phage isolation, characterization, and assays; partial technician effort for animal work. **Supplies:** bacterial culturing and resistance genotyping, phage purification and whole-genome sequencing, biofilm/catheter substrates, antibiotics and candidate nanoparticle adjuncts for Aim 2 screening. **Animals:** murine bacteremia model per-diem and procedures (Aim 3). **Other:** sequencing core fees, publication, and travel. No major equipment is requested. Figures are illustrative and to be finalized with institutional budgeting.

Vertebrate Animals

Animal work is proposed in Aim 3. A murine carbapenem-resistant ECC bacteremia model will assess cocktail dose/timing efficacy, consistent with the murine ECC bacteremia models used in the allowed literature (Fu et al., 2025; Kuo et al., 2025) and the septicemic-mouse efficacy testing of Entelli-02 (Subedi et al., 2025). Procedures will follow IACUC-approved protocols with humane endpoints, appropriate anesthesia/analgesia, and the minimum group sizes ([ILLUSTRATIVE], set by power analysis) needed for rigorous, reproducible survival and bacterial-load endpoints. Species justification reflects the established use of mice for ECC bacteremia efficacy testing in the allowed literature. (Aim 2 catheter-biofilm work is entirely in vitro, consistent with Cieslik et al., 2025, and involves no animals.)

Human Subjects / Clinical Trial

Not applicable as a clinical trial. No human-subjects research or patient treatment is proposed under this R21; de-identified bacterial clinical isolates will be used in accordance with institutional human-subjects determinations. The project is explicitly designed to enable future clinical translation: investigational phage products for individual MDR patients have been administered in the U.S. under FDA emergency/expanded-access IND (eIND) frameworks (e.g., compassionate-use programs such as UC San Diego IPATH) with local IRB oversight. Should a future patient need arise during the project, any such treatment would proceed separately under an eIND with IRB approval, not as part of this exploratory research.

Team & Environment

Principal Investigator [Name, Institution] — phage biology/translational AMR lead; overall scientific direction. **Co-Investigator, Clinical Microbiology [Name, Institution]** — provides characterized U.S. MDR/carbapenem-resistant ECC isolates and resistance genotyping. **Co-Investigator, Infectious Diseases / Phage Therapy [Name, Institution]** — clinical-translation and eIND-pathway expertise (e.g., an IPATH-style compassionate-use program). **Research Scientist/Postdoc and Technician [Names]** — phage isolation, characterization, biofilm/resistance assays, and in vivo studies. **Environment:** BSL-2 microbiology and phage-production capacity, genomics/sequencing cores, an AAALAC-accredited animal facility, and collaborative ties to phage-sourcing networks (e.g., Phage Directory / Citizen Phage Library) and potential translational partners (BARDA, CARB-X). Roles are templated for completion with confirmed personnel and institutions.

References

1. Subedi D, Gordillo Altamirano F, Deehan R, Perera A, Patwa R, Kostoulias X, Korneev D, Blakeway L, Macesic N, Peleg AY, Barr JJ. Rational design of a hospital-specific phage cocktail to treat *Enterobacter cloacae* complex infections. *Nature Microbiology*. 2025;10(11):2702–2719. https://doi.org/10.1038/s41564-025-02130-4

2. Kuo HY, Bregente CJB, Thuy TTD, Hidrosollo JH, Cruz-Papa DMD, Gutierrez TA, Huang YT, Chuang YJ, Hsueh PR, Kao CY. Isolation and characterization of strictly lytic bacteriophages against carbapenem-resistant *Enterobacter cloacae* complex. *Microbiology Spectrum*. 2025;13(11):e0083525. https://doi.org/10.1128/spectrum.00835-25

3. Fu SY, Chen XZ, Yi PC, Gao J, Wang WX, Gu SL, Gao JH, Liu DX, Xu HF, Zeng Y, Hu CM, Zheng Q, Chen W. Optimizing phage therapy for carbapenem-resistant *Enterobacter cloacae* bacteremia: insights into dose and timing. *Antimicrobial Agents and Chemotherapy*. 2025;69(4):e0168324. https://doi.org/10.1128/aac.01683-24

4. Cieslik M, Wojcicki M, Migdal P, Grygiel I, Bajrak O, Orwat F, Gorski A, Jonczyk-Matysiak E. Fighting biofilm: bacteriophages eliminate biofilm formed by multidrug-resistant *Enterobacter hormaechei* on urological catheters. *Medical Microbiology and Immunology*. 2025;214(1):33. https://doi.org/10.1007/s00430-025-00844-0