Resistance Networks

Antibiotics are a foundation of modern medicine and have saved hundreds of millions of lives since their discovery. Every year, roughly one in three people worldwide rely on them to treat common infections such as urinary tract infections, ear infections, and pneumonia, and they are essential for the hundreds of millions who undergo surgery, receive chemotherapy, or need an organ transplant each year. The emergence of antibiotic-resistant infections is therefore a major and growing global public health threat.

The aim of *Resistance Networks* is to measure the extent to which resistance moves through bacterial networks and to design interventions that prevent or slow that progression. Doing so could extend the efficacy of existing antibiotics and protect new antibiotics under development.

Antibiotic resistance is typically detected only after it has become a clinical problem. By the time an infection is found to be untreatable, a resistant bacterium has been isolated and tested, and in rare cases, sent for genome sequencing. Recent shifts in our understanding of microbial evolution suggest resistance emerges much earlier, moving from the bacterial networks that exist in and around us into pathogens that ultimately cause clinical disease.[1–3]

Genomic analysis has revealed the evolutionary power of small transmissible circular pieces of DNA, called plasmids, that collect and concentrate antibiotic resistance genes (ARGs), and move them, cell to cell, through bacterial networks.[4,5] Some of these plasmid types specialize in shuttling antibiotic resistance genes — these so-called pARGs move from between cells through a process called conjugation.[6–8] In this process, bacteria acquire complex traits — including antibiotic resistance, virulence, and ecological fitness — by acquiring plasmids and other mobile genetic elements rather than evolving them *de novo.*[9,10]

Such is the story of one sub-lineage of *Escherichia coli* — known as H30-Rx, the dominant strain within the broader ST131 lineage — which has become one of the most antibiotic-resistant bacterial lineages ever observed.[11–15] ST131 accounts for a large share of extended-spectrum beta-lactamase (ESBL)-producing *E. coli* infections worldwide, and one genomic study found that 91% of the drug-resistant ST131 isolates carrying a key ESBL resistance marker belonged to the H30-Rx sub-lineage (a highly antibiotic-resistant strain of bacteria, which produces ESBL – an enzyme that destroys many common antibiotics).[12] H30-Rx did not evolve resistance slowly, one mutation at a time. It did it in two moments.

Between 1979 and 1984 one bacterial cell type, already resistant to fluoroquinolone, acquired a plasmid-carrying resistance to four new classes of antibiotics simultaneously (third-generation cephalosporins, trimethoprim, aminoglycosides, and sulfonamides).[11–15] Four drug classes of resistance in one acquisition. That made it resistant to five.

Then, around 1992, the same lineage acquired a second plasmid.[13,14] The plasmid landed in a bacterial background that was already fluoroquinolone-resistant and already capable of

spreading. The added resistance gave H30-Rx a new advantage wherever antibiotics suppressed competing bacteria. Other traits likely mattered as well — including increased transmissibility, gut or urinary-tract colonization and persistence, virulence, and the ability of the plasmid to persist in that bacterial host without an overwhelming fitness cost. But the timing is striking: after this acquisition, the subclone expanded rapidly. Within a decade, H30-Rx had been detected on every inhabited continent.[12]

This is not a story about a single unlucky sequence. The same pattern has played out at least twice before. A related strain, ST69, caused a urinary tract infection outbreak in California in 2001 and became globally endemic within a decade — its expansion traces directly to a plasmid acquisition around 1990.[13] Other bacterial strains and pathogens have parallel stories, resistance in *Klebsiella*, *Salmonella*, and other highly virulent *E. coli* have similar stories with global implications.[16–18]

This tells us something important: Antibiotic resistance does not spread only when resistant bacteria spread. It spreads when the genetic instructions for resistance move between bacteria — carried on plasmids that cross strain boundaries, species boundaries, and communities.[19–21]

The rate at which these bacterial lineages acquire resistance, the conditions that drive it, whether resistance can be transmitted, and if so, how we might intervene to slow progression, has never been directly measured. That is the gap this program is designed to close.

Resistance Networks seeks to measure, predict, and interrupt resistance gene transmission to slow the emergence of antimicrobial resistance. If successful, such slowing could have a beneficial effect similar to the introduction of an entirely new class of antibiotics. The focus is on antibiotic resistance genes (ARG) linked to the plasmids (p) that carry them. These linked units – referred to here as pARGs - move within and between bacterial communities, especially when antibiotic treatment creates the conditions that allow them to expand.[22–28]

The program aims to determine whether routine antibiotic use may create predictable windows in which pARGs expand within the bacterial network of one individual, enter new bacterial hosts, and become transmissible to other bacterial networks.[22,29] If those windows can be identified before resistance spreads to the bacterial pathogens that cause disease, antibiotic resistance can be addressed as an actionable epidemiological problem of resistance-gene carrier movement, transmission, and colonization.

Antimicrobial resistance (AMR) is a leading and growing cause of mortality worldwide. In 2021, bacterial infections were associated with 4.71 million deaths, including 1.14 million deaths directly attributable to antibiotic resistance.[30] Since 2000, the prevalence of third-generation cephalosporin (3GC)-resistant *E. coli* has increased by more than 8 percentage points, and if that rate continues, resistance-attributable *E. coli* deaths are projected to reach approximately 345,000 per year by 2050.[30,31] Without intervention, AMR-attributable deaths across all bacterial pathogens are projected to exceed 1.91 million annually by 2050.[30] *Resistance Networks* asks whether part of that rise can be slowed by treating plasmid-mediated resistance

as a transmissible system that can be measured, modeled, and interrupted.

The development of new antibiotics reduces relative risk by opening new treatment options – this is necessary. It is slow (up to 10 years) and expensive with median cost estimates of $1.5 billion per drug in the US.[32,33] And because resistance inevitably evolves, these new antibiotics are often held in reserve, which creates challenging market dynamics. Public Health and stewardship strategies are helping but can't keep up.[34] We need new strategies designed to control the emergence and transmission of antibiotic resistance in bacterial networks. This is important for existing antibiotics, but it is critically important for protecting new antibiotics.

What if we could use epidemiological methods to detect, model, design and test interventions that prevent or slow the movement and exchange of resistance in bacterial networks?

If antibiotic resistance is only a trait that accumulates under selection pressure, the sole lever is to reduce that pressure — which means changing prescribing habits across entire health systems. But if plasmid-mediated resistance is transmitted within bacterial networks inside the treated gut,[5,35] if it spreads to the bacterial network of household contacts, and propagates through community or global transmission networks — the intervention calculus changes entirely.[36–49] If this is the case, then a local action — protecting one individual's microbiome after treatment, blocking shedding at the point of amplification, or interrupting household transmission — can have compounding benefits across the entire bacterial network. This means that individual interventions could become public health tools, and targeted action in high-prevalence communities could drive global trajectories. The same logic that made vaccines and infection control transformative then applies here — but only if we address resistance in bacterial networks as something that transmits, crosses thresholds of transmission, and can be reined in with appropriate interventions.

***Resistance Networks* asks whether we can reframe the problem of antibiotic resistance from targeting resistant bacteria to an epidemiological problem within bacterial networks and in so doing, develop novel strategies that slow the spread of resistance itself.**

The key epidemiological parameter is R_0 — the basic reproductive number — the average number of new cases generated by one index case in a susceptible, well-mixed population. In practice, R_0 is inferred from a set of measurable parameters: shedding rates, transmission rates and pathways, and the pathogen's own replication rate.[50]

For example, take SARS-CoV-2 virus when it first spilled into a dense population with no immunity. We cannot see a virus, we can only see its consequences — which was the pneumonia-like disease identified in hospitals. The landmark early estimate, published in January 2020, placed R_0 at 2.2, derived from symptomatic case counts alone.[51] At that value, ten generations of unchecked transmission (R_0^{10}) would produce about 2,600 cases.

We know now that was not the full story.

Once molecular testing could identify cases that never produced symptoms, we learned that an estimated 86% of early cases were asymptomatic — and those invisible cases were driving an estimated 79% of newly detected cases.[52] With that transmission included, R_0 was revised upward to 5.7[53] — at this level the same ten generations would produce 36,000,000 cases, the scale that produced a pandemic.

R_0 is a theoretical quantity meant to capture a pathogen's biology in a given context to predict whether it will propagate through a population or die out. The threshold between these two outcomes is R_0=1, what we define as the epidemic threshold.

The COVID-19 story is illustrative, but most populations are neither fully susceptible nor well-mixed: immunity varies, contact is structured by household and community, and the infectious agent circulates through contact, or the environment, or a vector. More fundamentally, R_0 cannot be directly observed. Transmission events are invisible. What epidemiologists count are new cases — and from those counts, working backward, they reconstruct R_0.[50] Mass testing changed the COVID-19 pandemic not because it changed the virus, but because it finally made new cases countable.

The central hypothesis of *Resistance Networks* is that plasmid-mediated antibiotic resistance spreads within bacterial networks to pathogenic bacteria like a contagion — and can be controlled like one.

The key problem, however, in treating antibiotic resistance epidemiologically is that there is no measurement that can be used to count "cases", *i.e.,* new transmissions that we can use to estimate the R_0 of plasmid-mediated resistance. The events we would count, new plasmid acquisitions, occur inside the gut in bacterial populations and are too complex and too infrequent to observe directly.[23,54]

As such, what this program seeks to measure directly is a resistance gene moving into a new bacterial host — crossing from one chromosomal background to another inside the gut, or from one individual's microbiome to another's. Counting those events, against the population of bacteria at risk, is the plasmid equivalent of counting new transmissions — the empirical input from which R_0 is reconstructed and against which the model is validated.[15,39]

As with other populations, the bacterial population of the gut is neither well-mixed nor fully susceptible. Bacteria are spatially structured in biofilms and mucus layers, only a fraction of Enterobacterales are permissive recipients for any given plasmid, and antibiotic exposure reshapes that susceptible pool in real time. A new model must therefore account for the parameters that deviate from the theoretical ideal: the density and community composition of recipient bacteria, the spatial structure of the gut network, the fraction of the bacterial

population protected from plasmid acquisition, and how antibiotic pressure changes each of these — so that R_0 can be back-calculated from observed transmission counts with the same rigor that epidemiologists use to estimate reproductive numbers from case surveillance, adjusted for the structure of the real population.

For decades, quantification of plasmid spread has come from laboratory experiments typically with isolated strains of *E. coli* cultured in controlled conditions in broth or on agar plates.[55] However, to get to meaningful epidemiological methods we need to read the network transmissions directly from living gut samples, tracing resistance genes, their mobile carriers, their bacterial hosts, and their independent movement within and between the bacterial networks of individuals, at the temporal and genomic resolution needed to model the effects of antibiotic use in local populations.[23,56–59] For years, we lacked the tools to do so. It was a significant measurement barrier. That's changing.

Why now?

New antibiotics are being developed. Phage therapies, CRISPR-based engineering of the microbiome, and targeted bacteriocins are entering clinical pipelines.[60–63] These advances are necessary. But there is more we can do. To extend the effectiveness of treatments we have and make new treatments more durable, we need to measure, model, and slow the transmission of resistance.

To do so, requires knowing which resistance genes to target, when to act (in the days after antibiotic pressure, when plasmid shedding peaks and the gut microbiome is disrupted), and in whom (the high-shedding individuals whose gut amplification disproportionately seeds household and community transmission). An intervention that clears a resistant plasmid from one patient while leaving the transmission network intact does not prevent the next resistance wave — it postpones it. And new antibiotics deployed without this understanding face a similar fate: resistance evolving faster than we can protect them.

For the first time, single-cell and linkage-preserving sequencing applied longitudinally could make the resistance network and its evolution visible.[64] These new techniques can resolve complete circularized genomes and plasmids, revealing that natural microbial populations are not composed of single strains but of coexisting lineages linked by shared mobile gene pools.[13,54] Proximity-ligation methods that preserve DNA contacts inside intact cells can now barcode thousands of bacterial cells in a single pooled library, making it possible to link plasmids and mobile resistance elements to the bacterial chromosomes that carry them.[21,23] These and other advances mean that for the first time it is possible to study how microbiome populations respond to antibiotic treatment *in vivo* across multiple individuals. Today, we can do so at pilot scale, and with further optimization and cost reduction, it is possible to do so at epidemiological scales. Measuring these parameters, against the standing plasmid-carrying

population, is the empirical input needed to reconstruct an analogous R_0 for transmission in a plasmid-carrying population using the type of modeling framework epidemiologists use for infectious disease outbreaks.

To achieve the above, it will be necessary to densely sample the antibiotic-driven dynamics of resistance emerging in local bacterial networks, both within and between the bacterial networks of individuals, from longitudinal cohorts. The key measurements require sampling of the gut microbiome for specific plasmid carriers before, during, and after antibiotic exposure, paired with sampling of close contacts and relevant household or community environments. Longitudinal designs with sufficient genomic resolution would move the field beyond static, single-time-point observations and begin to determine how antibiotic pressure changes plasmid abundance, composition, host range, and transmissibility over time.

Once these plasmid dynamics can be measured and linked to bacterial hosts and individuals, these data could be used to build predictive models that identify high-risk plasmids that transmit rapidly or carry antibiotic resistance genes, high-risk microbiome states, and high-risk windows of transmissibility. Computational tools are now capable of integrating large-scale genomic and epidemiological data to infer key parameters that shape plasmid dynamics, including transfer rates, fitness costs, and host range. These data-driven models then, in turn, help identify the biological constraints that shape plasmid movement *in vivo* such as incompatibility, host fitness effects, and ecological interactions.[65–68] And mechanistic models embedded in multiscale network models would then create the ability to design and test new interventions intended to block or slow forward transmission.[69,70]

Finally, experimental model systems, including controlled *in vivo* animal systems that reproduce gut Enterobacterales ecology and contact transmission, have advanced to the point where they can now serve two critical functions.[55,71] First, by measuring outcomes in the same R_0 framework, they provide a direct test of model predictions before costly human interventional trials. Second, and equally important, they provide the controlled platform needed to test whether model-identified interventions can change R_0 — something that cannot be done in observational human cohorts.

This is the moment to make the foundational measurements at scale, build the models, and design new interventions.

Goal of the Program

The goal of the *Resistance Networks* program is to build an epidemiological model that can predict whether a plasmid carrying antibiotic resistance genes (pARG) has epidemic-like potential (R_0 >1) to spread within and between human bacterial networks during and after antibiotic use. The program seeks to achieve 80% balanced predictive accuracy to enable the design and testing of new individual and public health measures. Such a model would provide

a leading indicator of resistance trajectories and potentially enable a new signal of rising pARG before resistance reaches levels at which empiric therapies must change. Current approaches rely on antibiotic resistance surveillance as a tool for population-level resistance management; for example, changing empiric prescribing guidelines when *E. coli* resistance exceeds 20% in local isolates.[72]

If successful, the model-based intervention approaches developed in *Resistance Networks* could slow the emergence of resistance by as much as a factor of 2 or more. Doing so could avert more than 1,300 deaths due to antibiotic resistance per day, and more than a new antibiotic class targeting priority pathogens alone by 2050.[a] And if plasmids are a primary driver of resistance to new antibiotics, such approaches could offer strategies to extend the working life and change the market dynamics of new antibiotic drugs that follow.

The program has 3 primary thrusts.

- Thrust 1: Determine, at 80% balanced predictive accuracy, whether antibiotic-amplified plasmid-associated resistance genes (pARGs) exhibit epidemic-like transmission (R_0 > 1) within and between gut bacterial networks in response to antibiotic use. Identify the levers for intervention at each scale.
- Thrust 2: Advance cost-effective tools that enable single-cell sequencing technology to scale measurements of plasmid transmission.
- Thrust 3: Test *in silico*, model-guided, key parameters and interventions in controlled *in vivo* systems, targeting the dominant driver(s) identified by Thrust 1.

The program is focused on Enterobacterales — the gram-negative bacterial order at the top of the WHO priority list for AMR burden[75] — because this order shares a bacterial network with the healthy gut microbiome, persists and proliferates under antibiotic pressure, and exchanges plasmids across humans, livestock, wildlife, and companion animals globally.[26,36,37,48,56,76–78] Demonstrating that measurement and control of resistance in the network of bacteria responsible for the largest share of global AMR burden establishes a blueprint applicable to any pathogen where resistance travels on mobile genetic elements — including *Acinetobacter baumannii* and other priority pathogens that share the gut ecosystem with Enterobacterales.

[a] Projection based on *E. coli* and *Klebsiella pneumoniae*, the two priority Enterobacterales pathogens with sufficient surveillance data to model, with data from the Global Burden of Diseases Study in Murray 2022 (The Lancet 399:10325) and Ikuta 2022 (The Lancet 400:10369).[73,74] The drug comparator is the Naghavi 2024 gram-negative drug scenario (The Lancet 404:1199).[30]

Call for abstracts and proposals

Resistance Networks is soliciting abstracts and proposals for work over three (3) years across three (3) coordinated thrusts, each contributing to the program goals and described in detail below.

Individual teams are not expected to address all components. The program's strength comes from program-level integration of focused, agile teams with deep — and sometimes narrow — expertise, working toward one shared quantitative goal. Proposers should clearly relate their work to one or more of the program goals and thrust areas. Proposals addressing only specific components of the program should expect to coordinate closely with the teams addressing the others, as detailed in the thrust descriptions below.

Across selected projects, definitions, assay benchmarks, reference panels, sample handling, data formats, and model inputs will be harmonized to support comparison, integration, and validation. Wellcome Leap will facilitate cross-collaboration within and between thrusts — integral to the program's success — as teams make progress together toward this shared goal.

Proposals should describe data sharing approaches, privacy-preserving integration, sample sharing where appropriate, coordination across projects, including any foreseen limitations to doing so. Proposals that draw on cohorts or samples from lower-resource settings should describe how the work will be conducted in partnership — including equity in scientific leadership, benefit-sharing, and data and sample sovereignty. Proposals should also include clear year-one milestones.

Thrust 1: Determine, at 80% balanced predictive accuracy, whether antibiotic-amplified plasmid-associated resistance genes (pARGs) spread epidemically ($R_0 > 1$) within and between gut bacterial networks in response to antibiotic use. Identify the key parameters in the model as levers for intervention at each scale.

Thrust 1 will do for plasmid-mediated resistance what epidemiologists did for SARS-CoV-2 in early 2020: count new transmissions — between cells in bacterial networks within the gut and between individuals in close contact networks. Working with variation across focal communities that differ in antibiotic exposure and gut microbiome composition, Thrust 1 will estimate the transmission parameters that determine whether a pARG crosses the epidemic threshold $R_0 = 1$. The parameters that drive spread above the threshold will be tested experimentally and prioritized for intervention in Thrust 3. Thrust 1 links two coupled processes that predict if the analogous R_0 of a pARG is expected to be greater than 1 due to antibiotic usage: how efficiently a pARG spreads between bacterial cells inside an antibiotic-

treated gut (R_0-within), and how reliably it transfers to close contacts afterward (R_0-between). Antibiotic use drives both simultaneously.

Thrust 1A develops a multiscale epidemiological model that links these coupled processes. Thrusts 1B and 1C supply the parameter measurements the model needs to place a pARG above or below the epidemic threshold. Two precision standards apply: 1) Individual parameter measurements in Thrusts 1B and 1C must achieve ±30% precision — the level at which combined uncertainty in the Thrust 1A model inputs remains small enough for the integrated R_0 estimates to be usable; and 2) Calculations for R_0-within (Thrust 1B) and R_0-between (Thrust 1C) must achieve ±50% precision, which is sufficient to ensure that a pARG with a true R_0 greater than 2 is confidently placed above the epidemic-like threshold (95% CI lower bound >1). Proposals must describe how their measurement approach will achieve the parameter-level precision standard, and how they will adapt their study design in response to emerging results across the program.

Thrust 1B and 1C longitudinal cohorts for Thrust 1 activities will need to be co-located in the same research sites to enable linkage between within-gut amplification dynamics and between-individual microbial network transmissions at the level of a shared microbiome environment. Teams who are proposing to only Thrust 1B or 1C should describe to what degree they have flexibility in their proposed cohort sites to enable alignment. Detailed cohort and site requirements appear in the Appendix. Thrust 1C includes independent empirical validation of R_0 >1 using wastewater or other surveillance measures.

Thrust 1A: Build a multiscale epidemic threshold modeling framework, to predict, with ≥80% balanced predictive accuracy, whether any pARG is above the epidemic threshold R_0 =1.

Thrust 1A aims to build a dynamic, multiscale model that predicts whether antibiotic use results in specific pARG transmission within and between two bacterial networks. Key model parameters can be initially informed by experimental values from the published literature or analysis of existing genomic data. Proposals may draw on existing model classes that are suited to capturing the biology at each scale, for example:[79–81]

- Deterministic models that capture within-gut state changes — donors, susceptible recipients, protected fractions — and how antibiotic exposure alters those states to change conjugation opportunity, plasmid persistence, and loss;
- Stochastic models that represent rare plasmid acquisition, loss, establishment, and household transmission events; and,
- Agent-based or network models that capture heterogeneity in bacterial interactions within the gut[82] and contact structure across individuals, households, and sites.

Success in Thrust 1A requires a multiscale model with 80% balanced predictive accuracy of coupled components of R_0 under conditions of antibiotic use and resistance prevalence for each of five sites. Performers in Thrust 1A are expected to work with empirical data from Thrust 1B and 1C to identify missing model components or key parameters that improve accuracy. Proposers to Thrust 1A should describe how they would collaborate with proposers from Thrust 3 to align on parameters that should be targeted for validation and intervention.

Thrust 1B: Determine the values of R_0-within for each pARG in a microbiome under antibiotic pressure, with a 95% confidence interval of ±50% the point estimate.

Thrust 1B will calculate R_0-within from the new pARG transfer events per *susceptible cell* in the microbiome community and the pARG loss rate using longitudinal sampling of fecal samples taken before, during, and after antibiotic treatment. New transfers are defined as antibiotic-driven pARG transmission events *in vivo* by observing the number of new chromosomal background transfers (as opposed to clonal expansion of already-resistant lineages). The number of susceptible cells are inferred from the size and structure of the bacterial network (the density of connections between pARG-carrying cells, those that are susceptible to horizontal transfer, and those that are protected from pARG transmission).

Proposals should specify how transmission will be estimated, how R_0-within will be calculated, and how a 95% confidence interval (CI) of ±50% the point estimate will be obtained. Proposals should specify which parameters they will measure, how precision will be reached, and state the current uncertainty in each from literature.

Specifically, to achieve this goal, it is expected that approaches will need to:

- Track changes in plasmid carriage longitudinally — before, during, and for at least one month after antibiotic treatment.
- Accurately link antibiotic resistance genes to specific plasmids and elements associated with the specific bacterial chromosome in a single cell; proposals should demonstrate how precision in their approach compares to isolate-based long-read sequencing as the gold standard.
- Resolve (sequence the complete chromosome and co-resident plasmids) enough Enterobacterales cells per sample with preserved plasmid-to-host linkage to track changes in plasmid carriage before, during, and after antibiotic treatment. Proposals should justify their cell count and demonstrate sufficient power to detect new plasmid-host combinations at the frequency expected when R_0-within = 1. Given current long-read sequencing costs, teams should describe their deployment approach and how they plan to achieve the desired scale and/or how they will stage their approach in collaboration with Thrust 2 to improve cost and time efficiencies as the program progresses.

Of interest are machine learning approaches applied to existing Enterobacterales experimental data used to identify genomic signatures of plasmid host range,[83] and cells resistant to plasmid invasion through competition among plasmids.

Thrust 1C: Determine the values of R_0-between for each pARG under antibiotic pressure with a 95% confidence interval of ±50% the point estimate.

To calculate the R_0-between the microbiome networks of individuals, Thrust 1C will measure the degree and duration of pARG amplification by antibiotics in individuals (index cases). In addition, Thrust 1C will count the number of close contacts who's gut microbiomes were previously negative and that receive the identical index case pARG — distinguishing transmission from independent acquisition or background carriage.

Proposals should specify how transmission will be estimated, how R_0 will be calculated, and how a 95% confidence interval (CI) of ±50% the point estimate will be obtained. Proposals should specify which parameters they will measure, how precision will be reached, and state the current uncertainty in each from literature.

Specifically, to achieve this goal, it is expected that approaches will need to:

- Accurately link antibiotic resistance genes to their plasmid; proposals should demonstrate how precision in their approach compares to isolate-based long-read sequencing as the gold standard.
- Identification of resistance gene and its plasmid (the pARG) at a detection sensitivity of at least 1 in 10,000 Enterobacterales cells. This sensitivity is required to detect newly transmitted pARGs before antibiotic amplification raises their abundance in the recipient (estimated from the average number of bacterial cells in one gram of fecal matter in individuals who are Enterobacterales carriers).
- Establish temporal ordering: plasmid context absent in contact before the index antibiotic course, and present afterward.[84]
- Distinguish plasmid transmission within and between bacterial networks from clonal spread of a resistant bacterium or independent acquisition of the same resistance determinant.
- Proposals justify cohort size using the transmission rate without antibiotic use in high-income settings from the literature as a floor. Three recent studies[15,36,38] constrain the daily household transmission rate for ESBL-producing *E. coli* to 0.002–0.005/person/day in high-income, high-sanitation settings, in the absence of antibiotic treatment. Over a 7-day period, this rate corresponds to a transmission probability of approximately 1–3%

as a floor. At this floor, even 200 pairs may yield zero observed events — a result that is itself informative, ruling out transmission above 1.5% with 95% confidence. Table 1 shows expected transmission events and achievable precision across different scenarios of cohort size and transmission probability given estimates of antibiotic amplification and duration of pARG shedding.

Table 1. Cohort size scenarios for estimating plasmid-specific per-course transmission probability

Pairs	Per-course transmission probability	Expected events	Outcome	Precision (95% CI)
100	1%	~1	Rules out >3% if zero observed	NA
100	3%	~3	Rules out >3% if zero observed	NA
100	30%	~30	Rate estimable	±36%
200	1%	~2	Rules out >1.5% if zero observed	NA
200	3%	~6	Rate estimable	±80%
200	30%	~60	Rate estimable	±25%

Expected events = pairs × transmission probability. Precision = ±(1.96/√n) × 100; shown only where ≥5 events expected. Rule-out bound = 3/pairs (95% upper Poisson bound when zero events observed). The plasmid-specific per-course transmission probability is unmeasured in LMIC settings; the 1–3% reference range is extrapolated from high-income household ESBL-E cohorts[15,36,38] and rates in high-burden communities may be substantially higher.

Of particular interest are study designs that can partition variance in the between bacterial networks transmission rate from household-level sources — plasmid identity, contact intensity, and individual microbiome susceptibility — and site-level sources — antibiotic use, WASH infrastructure, and community contact network structure. This partitioning identifies which drivers of transmission are amenable to individual-level intervention versus community-level intervention, and feeds directly into the Thrust 1A model as the distinction between random and fixed effects across focal sites.

Empirical validation of Thrust 1A. An independent check on the threshold classification is available to teams with community surveillance capability. If a pARG is spreading — R_0-overall > 1 — the number pARG per individual and the number of gut carriers in the catchment grows over time, and that growth produces a sustained, increasing signal in community wastewater. Longitudinal wastewater sampling using the program's plasmid-linkage criteria can therefore confirm or challenge the model's classification without relying on the cohort data used to build it. Teams are encouraged to collect longitudinal pARG data from the same catchments and to establish whether plasmid types detected in gut commensals appear in matched clinical isolates at comparable prevalence[85]. Agreement between model prediction and wastewater

trend strengthens confidence in the threshold call; disagreement is equally valuable — it signals a gap in the model, the measurement, or both. Precision of detection of specific circulating pARGs is the same as listed above.

Thrust 2: Advance cost-effective tools that enable single-cell sequencing technology to scale measurements of plasmid transmission.

Thrust 2 aims to increase the speed and efficiency of developing scalable library preparations that link complete bacterial chromosome and plasmid sequences from the same individual bacterial cell on scales of 1,000 per complex microbiome sample. Such advances will make the core measurements in Thrust 1B more affordable at epidemiological scales: reading which resistance gene sits on which plasmid inside which bacterial host cell — as complete, circularized, repeat-resolved, strain level host–plasmid sequence pairs — across enough cells and individuals to estimate R_0 with decision-grade confidence (estimated 1,000 Enterobacterales cells).

Resistance Networks invites proposals that address two primary technological challenges.

First, long-read sequencing costs per cell for library preparation and sequencing. The cost per cell sequence is the primary driver since neither the cell count per sample nor the participant numbers can be traded away.

Second, using current approaches, library preparation is performed on individually cultured clones, making cost proportional to cell number and requiring growth of each cell independently. Encapsulation approaches that barcode high molecular weight DNA associated with single clones within droplets or similar compartments without amplification before long-read, high-depth library preparation could, alternatively, pool thousands of barcoded cells into a single library, collapsing per-cell preparation cost by orders of magnitude while retaining single-cell resolution.

Other approaches of interest include strategies utilizing hydrogel immobilization as in CAP-seq, or linked-long read sequencing approaches analogous to those developed for eukaryotic single-cell genomics cloud analyses[86] — provided they are adapted for bacterial cells and achieve the same level of single-cell resolution for plasmid-to-host linkage.

Proposals to develop approaches that achieve linkage of host strain genotype - plasmid genotype at 5- to 10-fold reduction in cost per cell would enable higher powered studies.

Success in Thrust 2 is a validated single-cell library preparation workflow that demonstrates all three of the following capability requirements on a defined reference panel of priority Enterobacterales pARG variants from the focal research sites:

- **Linkage accuracy:** ≥95% concordance with complete long-read sequencing for resistance gene–mobile element–plasmid–bacterial host assignments on a defined reference panel of Enterobacterales cells spanning priority pARG variants.[13] Linkage must be resolved for all plasmids present in a cell — not only those carrying resistance genes — and for mobile genetic elements including transposons and insertion sequences, particularly those carrying functional genes. Concordance must be demonstrated for the complete co-localized genetic cargo of each cell: resistance genes, their mobile element context, the plasmid or chromosome carrying that element, and the bacterial host chromosome.
- **Throughput and cost:** ≥1,000 Enterobacterales cells per fecal sample (e.g., 1 gram input) with retained plasmid-to-host linkage, across a set of 10 samples — one per individual — within one month using standardized protocols that allow cross-individual comparison, at a per-cell processing at 5 -10X reduced cost. (Enterobacterales enrichment prior to library preparation is permitted provided the relative abundances of cell types in the enriched material remain proportional to their representation in the original sample, as demonstrated by digital PCR, and linkage accuracy and rare variant detection targets are met on enriched material.)
- **Biobanking:** workflows must preserve composite cell pools from each sample — collected prior to library preparation — for biobanking under standardized conditions, enabling experimental validation and cross-program access for the duration of the program.

Proposals should demonstrate performance against all four criteria, not cost reduction alone.

Thrust 3: Test model-guided interventions in controlled *in vivo* systems that drive a measurable reduction in R_0 for pARGs.

Thrust 3 seeks to validate the models for plasmid-mediated transmission within and between bacterial networks inferred from the human *in vivo* data of Thrust 1, in a controlled experimental system: if the model is correct, targeting barriers at the transmission points identified should reduce R_0. The aim is to show that targeting a dominant driver of R_0-overall — e.g., within-gut amplification, conjugation rate, or between-individual transmission — can push R_0 below 1 in a controlled system, or, where transmission is already below threshold, reduce the probability of crossing it.

Because Thrust 1 and Thrust 3 are developed in parallel, the dominant driver in each focal community will not be established at the outset; it emerges from Thrust 1 over the course of the program. Proposals should therefore state the interventions they plan to test and the transmission point(s) each targets, grounded in current evidence for the likely drivers, and describe the extent to which their design can accommodate Thrust 1 results as they become available — re-prioritizing or redirecting which driver is targeted as the R_0 decomposition sharpens. The strongest proposals will commit to a concrete starting intervention while building in this flexibility, and will coordinate with Thrust 1A on which parameters to target as shared findings mature.

The model system must first be established and characterized — its resident Enterobacterales population, dominant variants, and their responses to relevant antibiotics — and then validated against the human data (from Thrust 1) before it is used to test interventions.

Notably, model system design creates an opportunity for additional benefits. The same plasmids that carry ESBL resistance in clinical Enterobacterales circulate through agricultural systems, with intense fecal shedding and environmental release creating potential transmission pathways back to human populations. Farm-associated animal systems can therefore serve both as the controlled *in vivo* platform the program requires and as a direct test of interventions that interrupt zoonotic transmission at the farm interface and protect the herd.

Teams should test model-guided interventions targeting at least one of the two mechanistic scales:

- *Within-gut interventions* target conjugation rate, the fraction of Enterobacterales protected from plasmid acquisition, or gut community structure during the post-antibiotic amplification window. These may include microbiome restoration, competitive exclusion by defined bacterial consortia,[87] conjugation inhibition, targeted removal, phage, or CRISPR-based approaches[63,88,89] — with clear safety and clearance plans.
- *Between-animal transmission interventions* target the exchange interval and shedding between bacterial networks, environmental release, or contact transmission. These may include management or environmental controls — temporary cohorting or separation of antibiotic-treated animals, pen or bedding changes, manure and slurry controls — timed to the transmission-competent window.

Thrust 3 is designed with two aims. The first is to demonstrate that engraftment of a human-derived Enterobacterales strain or pARG variant successfully colonizes the model system's native gut community, achieves detectable plasmid carriage in resident lineages through conjugation, and is measurable using the R_0 framework applied in Thrust 1. The second is to demonstrate effective intervention in at least one model-guided strategy that produces a

measurable reduction in at least one component of R_0-overall relative to no-intervention controls after antibiotic-associated amplification. Specifically, successful projects should demonstrate at least one of the following:

- a ≥10 percentage-point increase[b] in the fraction of gut Enterobacterales protected from plasmid conjugation;
- a ≥10-fold reduction in *in vivo* plasmid conjugation rate;[b] or
- a ≥10-fold reduction in fecal shedding, environmental release, or between-animal transmission of priority pARGs.[b]

All interventions must demonstrate that they do not increase priority pathogen abundance, introduce new mobile resistance elements, or durably disrupt protective gut microbiome function.

Of particular interest are proposals using swine systems.[90] The swine gut microbiome and Enterobacterales ecology more closely resemble human gut biology than those of poultry or ruminants — making swine the highest-fidelity proxy for human plasmid networks.[71,91] Proposals using other animal systems should demonstrate that the system can recapitulate the bacterial community structure and transmission dynamics through which human-relevant priority plasmids traffic, while maintaining the relevance to One Health (the interactions between natural, clinical, and agriculture health) and additional potential for protecting agricultural herds and interrupting zoonotic transmission.

[b] *The 10-fold threshold for shedding and transmission endpoints are above the measurement uncertainty achievable with the cohort sizes established in Thrust 1C — ensuring that effects reported as successful are distinguishable from measurement noise.*

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Appendix A: Focal research site specifications

Teams with access to established prospective or retrospective cohorts that meet the requirements below are strongly encouraged to apply. Cohort-holding teams must be legally able to share biological samples, sequence data, and linked epidemiological metadata with program analysis partners under appropriate data sharing and material transfer agreements.

Resistance Networks seeks teams who work in research sites that represent extremes of the expectations for where pARGs may be epidemic.

- High-burden sites have at least two of the following: Enterobacterales carriage above 50%;[87] community 3GC or amoxicillin-clavulanate use above 20 DDD/1,000/day; *E. coli* 3GC resistance above 30%. These are settings where plasmid amplification and spread are most likely to be self-sustaining and where R_0 measurement is most consequential.
- Low-burden sites have all parameters at low to moderate levels: community 3GC or amoxicillin-clavulanate use below 20 DDD/1,000/day; 3GC resistance below 30%. These sites provide the ecological contrast against which high-burden dynamics are compared and the model is validated across its full parameter range.

Sites that do not fit neatly into either category should describe their burden profile explicitly. The program values the full epidemiological range.

Cohort platform

- Enrollment target. Research sites must be ready and able to enroll more than 100 participants, with a subset serving as index patients for longitudinal sampling before, during, and after antibiotic treatment.
- High-transmission populations. Cohorts with populations likely to be especially susceptible to pARG spread are encouraged to apply — including maternal-infant cohorts,[92,93] individuals receiving antibiotics simultaneously within a household or institution, or populations embedded in large close-contact networks.
- Antibiotic classes. The program focuses on third-generation cephalosporins (3GCs) and amoxicillin-clavulanate — the two community antibiotic classes with the strongest evidence for amplifying ESBL-carrying plasmids in the gut. Additional classes may be included at specific sites with explicit justification for local priority plasmid representation.

All focal research sites must demonstrate:

- **Community engagement.** An established framework for recruiting and retaining participants across longitudinal sampling, with community participation in study

design, together with arrangements for local scientific leadership, benefit-sharing, and stewardship of data and samples by the originating institutions and communities, where applicable.

- **Clinical linkage.** A direct connection to primary care or community clinic infrastructure through which antibiotic-treated outpatients can be identified and enrolled quickly.
- **Laboratory and sample handling capacity.** Capability to process fecal samples for high-molecular-weight DNA extraction, Enterobacterales culture, and genomic library preparation — or a confirmed logistics pathway, including regulatory clearance and cold-chain capacity, for shipping processed material to a partner laboratory.
- **Biobanking.** Capacity to bank fecal samples, cultured isolates, and epidemiological metadata at each sampling timepoint for cross-program access and confirmatory analysis.
- **Data security.** A governance framework meeting local and international standards for human genomic and health data.
- **Wastewater and community surveillance access.** Teams should describe whether wastewater infrastructure is in place or accessible and whether existing clinical isolate collections and pARGs circulating in commensal bacteria can be linked to the cohort. Wastewater access is not a requirement for site selection, but community-level pARG surveillance is an expected output of the program.