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ASSESSING ASSOCIATIONS BETWEEN EVOLVED BETA LACTAM RESISTANCE PROFILES AND GENTAMICIN SUSCEPTIBILITY IN ESCHERICHIA COLI LINEAGES

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Abstract:

This analysis examines whether evolved resistance profiles identify collateral susceptibility advantages relevant to antibiotic treatment design. Secondary experimental data published in 2019 cover 60 Escherichia coli lineages assessed after ten passages under cefotaxime selection. Parental concentration normalization, multiple linear regression with HC3 standard errors, Spearman correlations and individual omission analyses distinguish conditional associations from ancestral susceptibility changes. A doubling of cefotaxime resistance is associated with an 8.7% lower fitted geometric mean gentamicin concentration, with a 95% confidence interval indicating reductions from 3.8% to 13.3%. The cefotaxime coefficient remains negative across all individual omission analyses. Replacing source estimates with technical medians leaves results unchanged. Nevertheless, only one lineage exhibits a gentamicin concentration below the parental benchmark. The evidence therefore distinguishes relative susceptibility gradients among evolved lineages from restored ancestral sensitivity. This distinction refines evolutionary trade off interpretation without attributing associations to unmeasured biological mechanisms. For antimicrobial research, the findings support evaluating conditional resistance relationships alongside ancestral benchmarks before prioritizing drug sequences or combinations for experimental validation.

Key Words: Antimicrobial Resistance; Collateral Sensitivity; Escherichia Coli; Evolutionary Tradeoffs; Multiple Regression

1. Introduction:

An inverse association between resistance to two antibiotics does not necessarily mean that evolution has restored susceptibility to either drug. This distinction matters for strategies that seek to exploit collateral sensitivity, where adaptation to one antibiotic increases susceptibility to another. A lineage may remain less susceptible than its ancestor while showing greater susceptibility than other evolved lineages. Treating these comparisons as equivalent can overstate the therapeutic meaning of experimental resistance profiles. This analysis examines that distinction through the relationships between three beta lactam resistance phenotypes and gentamicin susceptibility in experimentally evolved Escherichia coli. Evolutionary tradeoffs provide a theoretical basis for these relationships. Resistance adaptations can alter enzyme activity, membrane permeability, drug transport and cellular resource allocation, producing consequences beyond the selecting antibiotic. However, these consequences need not impose a consistent susceptibility cost. Andersson and Hughes, 2010, explain that the fitness consequences of resistance depend on the mutation, genetic background and environment. Lázár et al., 2013, demonstrated that physiological changes associated with aminoglycoside resistance can increase susceptibility to other antibiotics. Their findings establish a mechanism for linked drug responses without implying that collateral effects are reciprocal or uniform across antibiotic pairs.

The closest experimental literature distinguishes the existence of collateral responses from their repeatability and usefulness. Imamovic and Sommer, 2013, mapped collateral sensitivity networks to inform antibiotic cycling. Munck et al., 2014, examined how responses to component drugs relate to resistance development under combinations. These approaches show why resistance profiles can inform treatment design, but they address different exposure regimes. Evidence from selection with a single antibiotic cannot automatically establish the consequences of simultaneous combination treatment. Suzuki et al., 2017, demonstrated this boundary by identifying combinations that either suppressed or accelerated resistance development. Variation among evolutionary pathways further limits simple predictions. Barbosa et al., 2017, observed contrasting collateral responses among replicate populations selected with the same antibiotic. Nichol et al., 2019, directly examined this contingency in Escherichia coli and showed that divergent evolutionary outcomes can produce heterogeneous responses to subsequent drugs. Together, these findings challenge the assumption that resistance magnitude alone identifies a predictable collateral susceptibility advantage. They also motivate separating the response to the selecting antibiotic from responses expressed across related antibiotics.

The reviewed evidence does not establish whether cefotaxime, cefazolin and ceftolozane/tazobactam resistance provide distinct conditional information about gentamicin response within the endpoint sample examined here. Cefotaxime resistance measures adaptation against the selecting antibiotic. Cefazolin resistance captures a collateral response to another cephalosporin, while ceftolozane/tazobactam resistance measures response to a beta lactam combined with an enzyme inhibitor. Their shared pharmacological context can generate overlapping phenotypic variation, but it does not make them interchangeable measures. The relevant empirical question is whether an association observed for one resistance phenotype persists when the other two enter the same model. Gentamicin response requires equally careful interpretation. The outcome is the base two logarithm of each lineage's minimum inhibitory concentration relative to the corresponding parental estimate. Higher values indicate lower susceptibility, while values below zero indicate greater susceptibility than the parent. This normalization distinguishes differences among evolved lineages from susceptibility changes relative to their common ancestor. It does not classify clinical susceptibility or measure treatment effectiveness. Pál et al., 2015, emphasize that collateral responses depend on the physiological consequences of

adaptation, while Allen et al., 2021, demonstrate that local environmental conditions can modify their expression. These considerations make the parental benchmark and assay setting essential to interpretation.

This study aims to determine how evolved beta lactam resistance profiles relate to gentamicin susceptibility across Escherichia coli lineages. The central research question asks whether cefotaxime, cefazolin and ceftolozane/tazobactam resistance retain distinct associations with gentamicin response after accounting for their shared variation. The analysis examines each relationship separately through rank correlations and jointly through regression. It also evaluates whether individual lineages or an alternative summary of technical measurements change the estimates. These objectives match the observed phenotypes without imposing an unmeasured moderator or biological pathway. The empirical setting comprises 60 Escherichia coli DH10B evolutionary lineages exposed to cefotaxime and assessed after passage ten in the experiment reported by Nichol et al., 2019. The data form an endpoint cross section within the eligible evidence period from 2010 to 2023. Ordinary least squares with HC3 standard errors estimate conditional associations among normalized resistance measures. Spearman correlations describe unadjusted monotonic relationships, while individual omission analyses and technical median comparisons assess sensitivity. The common experimental setting enables comparison across lineages under the same selection protocol, although endpoint associations do not identify causal effects or establish transferability to clinical populations.

The findings distinguish a relative susceptibility gradient from an ancestral susceptibility advantage. Cefotaxime resistance retains an inverse conditional association with gentamicin concentration, whereas the estimates for cefazolin and ceftolozane/tazobactam remain imprecise. Nevertheless, only one lineage has a gentamicin concentration below the parental estimate. The contribution is therefore an interpretation of resistance profiles that combines conditional association with an explicit ancestral benchmark. For laboratory evaluation, this distinction helps prevent an inverse relationship among evolved phenotypes from being treated as sufficient evidence for an effective antibiotic sequence or combination. Its relevance extends to experimental systems using comparable measurements, while treatment implications remain bounded by the observed strain, exposure regime and assay conditions.

2. Theory, Conceptual Framework and Research Question Development:

This analysis draws on evolutionary trade off theory to examine how resistance acquired under antibiotic selection relates to susceptibility to another antibiotic. Selection favors bacterial variants that survive exposure, but the resulting adaptations can alter several physiological functions. Resistance therefore develops within an interdependent system of drug targets, membrane transport, enzyme activity and cellular resource allocation. Andersson and Hughes, 2010, explain that resistance can impose fitness costs, although compensatory evolution can reduce those costs. This theoretical setting supports examining linked resistance phenotypes without assuming that stronger resistance necessarily produces greater susceptibility elsewhere. Collateral sensitivity occurs when adaptation to one antibiotic increases susceptibility to another. Cross resistance describes the competing outcome, where adaptation reduces susceptibility to additional antibiotics. Lázár et al., 2013, demonstrated that changes in membrane energetics and drug efflux can connect resistance to one antibiotic with increased sensitivity to others. Their findings establish a physiological basis for collateral effects, but they do not establish that these effects operate equally in both directions. Accordingly, this analysis treats gentamicin susceptibility as an empirical outcome rather than a presumed consequence of beta lactam resistance.

The framework uses observed resistance phenotypes from 60 Escherichia coli DH10B evolutionary lineages exposed to cefotaxime and assessed after passage ten. Cefotaxime, cefazolin and ceftolozane/tazobactam resistance form three distinct explanatory measures. Gentamicin susceptibility provides the outcome. Each measure expresses the base two logarithm of the lineage minimum inhibitory concentration divided by the corresponding parental concentration. For gentamicin, a higher value indicates lower susceptibility. These measures describe phenotypic variation across lineages and do not constitute a latent resistance construct. Cefotaxime resistance captures adaptation to the antibiotic that imposed the experimental selection pressure. Its magnitude reflects the resulting ability to tolerate cefotaxime, but different evolutionary routes can produce similar resistance levels. Enzyme changes may primarily affect beta lactam activity, whereas broader physiological changes may also influence gentamicin susceptibility.

Consequently, cefotaxime resistance alone does not imply a uniform collateral response. An association with gentamicin susceptibility would indicate that adaptation to the selecting antibiotic carries information about another drug response. An absent association would indicate that cefotaxime resistance magnitude does not consistently distinguish gentamicin responses across these lineages. Nichol et al., 2019, demonstrated divergent resistance trajectories and heterogeneous collateral responses in the experiment supplying these observations. Their findings support a research question that permits positive, negative or absent associations rather than a fixed directional hypothesis.

RQ1: What association exists between cefotaxime resistance and gentamicin susceptibility across evolved Escherichia coli lineages after passage ten?

Cefazolin resistance captures response to another cephalosporin following cefotaxime selection. Unlike cefotaxime resistance, it represents a collateral phenotype rather than adaptation measured against the selecting antibiotic. Its inclusion distinguishes resistance expressed across related drugs from resistance specific to cefotaxime. The shared beta lactam context makes cefazolin resistance informative about the breadth of the evolved phenotype. However, gentamicin acts through a different cellular target. Shared resistance across cephalosporins therefore does not establish equivalent resistance to gentamicin. The association depends on whether the underlying adaptations affect functions relevant to both drug groups. Pál et al., 2015, explain that collateral responses arise from the physiological consequences of resistance mechanisms rather than drug classification alone. This distinction supports examining whether cefazolin resistance contributes information about gentamicin susceptibility beyond the selecting drug phenotype.

RQ2: What association exists between cefazolin resistance and gentamicin susceptibility across evolved Escherichia coli lineages after passage ten?

Ceftolozane/tazobactam resistance measures response to a beta lactam combined with an enzyme inhibitor. The source experiment assessed this combination at a fixed ratio. Its resistance phenotype therefore differs from those measured using

cefotaxime or cefazolin alone. This measure captures bacterial growth inhibition under the combined action of the antibiotic and inhibitor. It does not separately identify enzyme activity, membrane transport or regulatory changes. Its relationship with gentamicin susceptibility may consequently converge with, or diverge from, the relationships observed for the other beta lactam measures. Barbosa et al., 2017, demonstrated that alternative evolutionary routes can generate contrasting collateral responses, including changes in aminoglycoside sensitivity among beta lactam adapted populations. That evidence comes from Pseudomonas aeruginosa and supports mechanistic contingency rather than a transferable directional prediction for Escherichia coli.

RQ3: What association exists between ceftolozane/tazobactam resistance and gentamicin susceptibility across evolved Escherichia coli lineages after passage ten?

The conceptual framework links three observed beta lactam resistance measures to one observed gentamicin response. The links represent associations across evolutionary lineages, not causal effects of changing one resistance phenotype. The research questions remain exploratory and allow each relationship to differ in direction or show no detectable association. The lineage constitutes the analytical unit, while technical replicate measurements support the source concentration estimates rather than additional independent observations.

3. Data:

This analysis establishes the provenance of the experimental observations, documents sample construction and verifies the measures used to examine resistance associations. The data preserve lineage identifiers, original concentration estimates and reproducible transformations across a common experimental endpoint.

3.1 Data Sources, Population, and Sample Construction:

The empirical source is the experimental dataset accompanying Nichol et al., 2019, published in Nature Communications. The authors evolved 60 Escherichia coli DH10B populations carrying an SHV 1 beta lactamase construct under cefotaxime selection. The analysis uses the Passage 10 worksheet in Supplementary Table 2.xls, distributed through the authors' Collateral Sensitivity Repeatability repository. That worksheet contains one parental reference and 60 evolved lineages, identified as X1 through X60. The publication provides the experimental protocol, antibiotic definitions and measurement procedures. The workbook identifies the source file and worksheet but does not preserve a versioned release, commit identifier or original extraction timestamp. The publication carries a Creative Commons Attribution 4.0 licence, while the repository contains an MIT licence for its software and associated documentation. These permissions require preservation of the relevant attribution and notices. The analytical unit is an independently evolved bacterial lineage assessed after passage ten. The data form an experimental endpoint cross section. They contain neither annual observations nor repeated endpoint measurements for the same lineage. The publication falls within the eligible literature period from 2010 to 2023, but that interval does not represent the observation frequency. The protocol comprises ten passages of approximately 24 hours each; exact experimental calendar dates are not recorded in the analytical workbook. Minimum inhibitory concentrations describe endpoint phenotypes rather than flows, stocks or period averages.

The target population comprises resistance phenotypes arising under comparable laboratory conditions. The accessible sampling frame consists of the 60 evolved lineages reported in the source experiment. All were retained through complete enumeration. The parental strain supplies normalization benchmarks and does not enter the association sample as an additional evolved lineage. The population of clinical isolates, patients or naturally circulating Escherichia coli strains is outside this sampling frame. Experimental context defines the interpretation of these observations. Imamovic and Sommer, 2013, examined collateral response networks following selection with individual drugs, whereas Munck et al., 2014, examined resistance development under drug combinations. Suzuki et al., 2017, further demonstrated that combination exposure can suppress or accelerate resistance development. Maltas and Wood, 2019, characterized collateral profiles in another bacterial species. These differences justify retaining the present dataset as a distinct experimental sample rather than pooling observations across incompatible selection protocols.

Table 1: Sample construction

Stage	Units	Observations	Reason or Rule
Original endpoint worksheet	61 bacterial records	61	One parental reference and 60 evolved lineages
Parental reference excluded from association sample	1 reference	1	Used only as the normalization denominator
Eligible evolved sample	60 lineages	60	X1 through X60 at passage ten
Duplicate identifier exclusions	0	0	No repeated lineage identifiers
Missing measurement exclusions	0	0	All four selected concentration estimates are available
Invalid denominator exclusions	0	0	All parental concentrations are positive
Final joint regression sample	60 lineages	60	Complete observations for three predictors and one outcome
Final correlation sample	60 lineages	60 per correlation	Identical coverage across all three comparisons

The sample boundaries follow the supplied conceptual framework and endpoint worksheet. No eligibility rule was changed after inspecting association estimates. There are no losses from external matching, missing controls, lag construction or differencing.

3.2 Data Integration, Cleaning, and Quality Assurance:

The analytical workbook separates original measurements, calculated variables, definitions and source documentation. Each analytical row represents one lineage, and each column represents one measurement or identifier. This organization follows the principles of explicit spreadsheet structure described by Broman and Woo, 2018, and consistent observational structure described by Wickham, 2014. Integration used the lineage identifier to align the analytical worksheet with the original endpoint worksheet. For each lineage, the maximum likelihood concentration estimates for cefotaxime, cefazolin, ceftolozane/tazobactam and gentamicin were matched to their corresponding source fields. The relationship was one analytical record to one source record. All 60 lineages matched. The audit identified no duplicate identifiers, unmatched lineages, conflicting selected estimates or increases in the number of observations.

The original worksheet contains three technical concentration measurements for each antibiotic and lineage. The analysis uses the authors' maximum likelihood estimate rather than treating these measurements as independent bacterial populations. This distinction preserves the experimental unit and avoids artificial inflation of the sample size, consistent with Vaux et al., 2012. All four selected concentration estimates and their parental denominators were checked for missingness, numerical validity and positive values. No selected analytical value was missing, zero or negative. The original worksheet contains nine missing parental test probabilities, one for each antibiotic. These entries are structurally inapplicable because the parent is the comparison reference. They were preserved and did not affect construction of the analytical variables. No missing value was replaced with zero, imputed or interpolated. Every selected lineage concentration matched the corresponding source estimate. Independent recalculation reproduced all four logarithmic measures exactly. These checks preserve an auditable connection between source values and derived quantities, consistent with Sandve et al., 2013. No observation was removed or winsorized because of its magnitude. Extreme concentrations remained in the sample when they matched the source record. No external dataset, currency adjustment or frequency conversion was required. The analysis uses the reported experimental endpoint and makes no claim to reconstruct information available during earlier passages. Preserving original values separately from calculations supports computational reproducibility as described by Peng, 2011.

3.3 Variable Measurement, Construction, and Validation

The explanatory variables are observed resistance phenotypes for cefotaxime, cefazolin and ceftolozane/tazobactam. The outcome is the gentamicin response relative to the parental strain. The distinction matters because resistance costs and collateral susceptibility are related concepts but are not interchangeable. Andersson and Hughes, 2010, describe resistance costs as context dependent, while Lázár et al., 2013, demonstrate that resistance adaptations can alter susceptibility to other antibiotics. For each antibiotic, the implemented transformation is:

$$Z_{id} = \log_2 \frac{M_{id}}{M_{0d}}$$

In Equation 1, Z_{id} denotes the normalized response of lineage i to antibiotic d, M_{id} denotes its endpoint maximum likelihood minimum inhibitory concentration, and M_{0d} denotes the corresponding parental estimate. The lineage index covers 60 evolved populations. The antibiotic index identifies the four selected drug measurements. The parental concentrations are 0.25 micrograms per millilitre for cefotaxime, 128 for cefazolin, 2 for ceftolozane/tazobactam and 0.25 for gentamicin. Each denominator is specific to its antibiotic. Normalization therefore expresses change relative to the common parent without equating absolute concentrations across drugs. A transformed value of zero denotes the same concentration as the parent. A value of one denotes a twofold increase, while a value of negative one denotes a halving. Higher predictor values indicate greater resistance relative to the parent. Higher gentamicin outcome values indicate lower susceptibility. Consequently, a negative association with the outcome corresponds to greater gentamicin susceptibility as the predictor increases.

Table 2: Variable dictionary

Variable	Analytical Role	Definition and Construction	Source and Identifier	Unit and Frequency	Transformation and Direction	Missing Value Rule	Coverage and Limitation
Lineage	Analytical identifier	Original population identifier	Endpoint worksheet, Lineage	One record per lineage	None	None missing	60 experimental populations
Cefotaxime resistance	Predictor	Endpoint estimate divided by parental estimate of 0.25	CTX_mle	Concentration in micrograms per millilitre at passage ten	Base two logarithm; higher means greater resistance	None missing; no imputation	60 lineages; selecting antibiotic
Cefazolin resistance	Predictor	Endpoint estimate divided by parental estimate of 128	CFZ_mle	Concentration in micrograms per millilitre at passage ten	Base two logarithm; higher means greater resistance	None missing; no imputation	60 lineages; collateral phenotype
Ceftolozane / tazobactam resistance	Predictor	Endpoint estimate divided by parental estimate of 2	CFT_mle	Concentration in micrograms per millilitre at passage ten	Base two logarithm; higher means greater resistance	None missing; no imputation	60 lineages; source uses a fixed 2:1 drug ratio
Gentamicin response	Outcome	Endpoint estimate divided by parental estimate of 0.25	GNT_mle	Concentration in micrograms per millilitre at passage ten	Base two logarithm; higher means lower susceptibility	None missing; no imputation	60 lineages; laboratory response rather than clinical classification

The three explanatory measures remain separate. No weighting, aggregation or composite resistance score was imposed. Pál et al., 2015, emphasize that collateral responses depend on resistance mechanisms, making a single interchangeable resistance scale difficult to justify. Barbosa et al., 2017, further show that different evolutionary routes can produce contrasting collateral effects. Validation therefore focused on source reconciliation, denominator consistency, positive concentrations and exact reproduction of transformations. Factor analysis and internal consistency coefficients were not applied because the measures are observed drug responses rather than reflective indicators of a latent construct. Environmental transferability also remains bounded by the assay setting, consistent with evidence from Allen et al., 2021, that local conditions can modify collateral responses.

Table 3: Descriptive statistics for normalized measures

Variable	Valid Count	Mean	Standard Deviation	Median	Minimum	Maximum
Cefotaxime resistance	60	5.567	2.902	4.000	2.000	10.000
Cefazolin resistance	60	3.717	0.825	4.000	2.000	5.000
Ceftolozane/tazobactam resistance	60	2.883	0.976	3.000	1.000	5.000
Gentamicin response	60	0.550	0.565	1.000	Negative 1.000	2.000

Gentamicin concentrations equal 0.125 micrograms per millilitre in one lineage, 0.25 in 26, 0.5 in 32 and 1.0 in one. Thus, one lineage has a lower estimated concentration than the parent, 26 have the same estimate and 33 have higher estimates. These counts describe relative concentration changes and do not impose clinical susceptibility breakpoints.

3.4 Analytical Framework, Identification, and Reproducibility:

The analysis is exploratory and associational. It examines whether the three beta lactam resistance measures contain distinct information about gentamicin response across lineages exposed to the same experimental selection regime. The implemented baseline specification is:

$$Y_i = \alpha + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{3i} + \varepsilon_i$$

In Equation 2, $Y_i$denotes normalized gentamicin response, X_{1i}cefotaxime resistance, X_{2i}cefazolin resistance and X_{3i} ceftolozane/tazobactam resistance. All four variables use Equation 1. The intercept is α, the conditional association coefficients are β_1, β_2 and β_3, and $\varepsilon_i$denotes unexplained variation. Ordinary least squares was fitted to all 60 observations with an intercept and three predictors. All measures refer to passage ten. No additional controls, interactions or fixed effects were included. The design matrix has full rank, and the predictor variance inflation factors are 2.431, 1.086 and 2.555, respectively. Inference used HC3 standard errors, which adjust residual contributions for leverage, with approximate Student t reference distributions using 56 residual degrees of freedom. Mansournia et al., 2021, explain the role and limitations of robust variance estimation. Imbens and Kolesár, 2016, show that robust inference can remain sensitive to finite sample conditions. Accordingly, these standard errors address unequal error variance under independent lineage sampling but do not establish independence or remove model misspecification.

The coefficients describe partial linear associations conditional on the other two resistance measures. Their causal interpretation is not identified because all phenotypes were measured at the same endpoint and may share unmeasured genetic or physiological determinants. Common ancestry and experimental conditions also restrict generalization beyond the source system.

Two exploratory checks examined specific analytical threats. Spearman correlations assessed whether the associations depended on the linear scale and were calculated on the same 60 lineages, following the distinction between linear and monotonic association explained by Schober et al., 2018. Sixty additional regressions each omitted one lineage to examine sensitivity to individual observations; every such fit retained 59 lineages. These checks did not change eligibility rules or the baseline sample.

The research questions and analytical choices were not preregistered. Estimates therefore remain exploratory. Interpretation considers coefficient direction, magnitude and uncertainty rather than treating a probability threshold as evidence of biological importance, consistent with Wasserstein et al., 2019. An inconclusive coefficient does not establish the absence of an association, as Greenland et al., 2016, explain. The workbook preserves the original endpoint observations, source row identifiers, parental benchmarks, variable dictionary and calculation formulas. Common lineage identifiers, endpoint timing and normalization rules align the four measures. Reproducibility is strongest for calculations from the supplied workbook; the absence of a pinned repository version limits reconstruction of its original download history.

4. Methods:

4.1 Research Design and Identification Strategy:

This analysis uses an exploratory associational design to examine relationships between evolved beta lactam resistance profiles and gentamicin susceptibility. The unit of analysis is an Escherichia coli evolutionary lineage assessed after passage ten of cefotaxime selection. Useful variation arises from differences in endpoint resistance phenotypes across 60 lineages sharing a parental background and experimental selection protocol. The source experiment and its measurement procedures are documented by Nichol et al., 2019. The estimands are partial linear associations between each resistance measure and normalized gentamicin response, conditional on the other two resistance measures. The analysis also estimates unadjusted monotonic associations to distinguish pairwise patterns from relationships conditional on the wider resistance profile. These comparisons answer whether the three phenotypes provide distinct information about gentamicin response within the observed experimental sample. The design does not identify causal effects of one resistance phenotype on another. All selected measurements refer to the same endpoint, and unmeasured genetic or physiological changes may jointly determine them. A common experimental protocol improves comparability but does not remove this source of confounding. The estimates therefore describe associations among evolved lineages rather than effects of assigning antibiotic treatments.

4.2 Sample, Measurement Alignment, and Model Specification:

Table 1 documents sample construction, and Table 2 defines the analytical variables. The estimation sample includes all 60 evolved lineages with complete measurements. The parental record supplies normalization denominators and is excluded as an additional regression observation. Technical triplicates support each concentration estimate but are not treated as independent lineages, consistent with the distinction between biological replication and repeated measurement explained by Vaux et al., 2012.

Equation 1 defines the implemented transformation: the base two logarithm of each endpoint minimum inhibitory concentration divided by its antibiotic specific parental estimate. The predictors represent cefotaxime, cefazolin and ceftolozane/tazobactam resistance. The outcome represents normalized gentamicin concentration. Higher predictor values indicate greater resistance, whereas higher outcome values indicate lower susceptibility. A one-unit predictor increase corresponds to a doubling of its concentration relative to the parent. The baseline specification is Equation 2, comprising an intercept and the three resistance predictors. All variables enter contemporaneously at passage ten. No additional controls, interactions, lags, weights or fixed effects were included. The three predictors remain separate observed phenotypes rather than components of a composite index. Each

coefficient consequently describes the association with gentamicin response while holding the other two phenotypes constant. The outcome occupies four observed logarithmic levels. Linear regression provides a summary of conditional mean differences on that scale rather than a complete model of the outcome distribution. For interpretation on the concentration scale, raising two to a coefficient gives the fitted multiplicative contrast associated with a doubling of the corresponding predictor. This contrast concerns the fitted geometric mean concentration and does not represent a clinical susceptibility classification.

4.3 Data Processing, Estimation, and Inference:

Processing began with the analytical and original measurement worksheets in the supplied workbook. Lineage identifiers linked each analytical record to its source record. The audit checked identifier uniqueness, complete matching, numerical agreement of selected concentration estimates, positive parental denominators and exact reproduction of logarithmic transformations. All selected measurements were available, so estimation required no deletion, imputation or interpolation. Source consistent extreme observations were retained without winsorization. Ordinary least squares used the same 60 observations for all three predictors. The coefficient vector was calculated from the full rank design matrix using the inverse cross product solution. HC3 standard errors addressed possible unequal error variance while adjusting residual contributions for leverage. The implemented covariance estimator was:

$$\widehat{\mathrm{Var}}_{\mathrm{HC3}}[\hat{\beta}] = A\left[\sum_{i=1}^{n} \frac{\hat{\varepsilon}_i^{\,2}}{[1-h_i]^2} x_i x_i'\right] A, \qquad A = [X'X]^{-1}$$

In Equation 3, $\hat{\beta}$ contains the estimated intercept and three slopes, X is the design matrix, and $x_i$contains the intercept and predictor values for lineage i. The fitted residual is $\hat{\varepsilon}_i$, leverage is $h_i = x_i' A x_i$, and n equals 60. The prime denotes matrix transposition. Standard errors are the square roots of the covariance matrix diagonal. Two-sided probabilities and 95% confidence intervals use an approximate Student t reference distribution with 56 residual degrees of freedom. Mansournia et al., 2021, explain how robust variance estimators address heteroskedasticity and leverage without correcting all forms of model misspecification. Imbens and Kolesár, 2016, further establish that robust inference requires caution in finite samples. Inference treats evolutionary lineages as independent analytical units. No cluster adjustment was applied because the supplied dataset does not identify multiple experimental clusters suitable for estimating a separate cluster covariance structure. Shared experimental influences remain a limit to that independence assumption. HC3 does not correct dependence arising from unrecorded common influences. Calculations used Python 3.12.14, pandas 2.2.3, NumPy 2.3.5 and SciPy 1.17.0. NumPy performed matrix calculations, while SciPy supplied statistical distributions, rank correlations and residual normality testing. All implemented procedures were deterministic and required no random seed or iterative optimization.

4.4 Diagnostics, Sensitivity Analysis, and Reproducibility:

Diagnostics addressed predictor overlap, residual distribution, variance patterns and observation sensitivity. Variance inflation factors were calculated by regressing each predictor on the remaining predictors with an intercept. Each factor equals the reciprocal of one minus the auxiliary regression coefficient of determination. Full matrix rank was also verified. These checks assessed precision and redundancy without using a threshold to remove predictors. The Shapiro Wilk test assessed fitted residual normality rather than normality of the original concentration measures. A Breusch Pagan diagnostic regressed squared baseline residuals on the intercept and three predictors. Its statistic equalled the sample size multiplied by the auxiliary coefficient of determination and was compared with a chi square distribution having three degrees of freedom. Neither diagnostic determined sample inclusion or replaced the HC3 inference procedure.

Spearman correlations examined unadjusted monotonic relationships between each predictor and the outcome. Tied values received average ranks, and approximate two-sided probabilities were obtained through SciPy. These correlations used the same 60 lineages as the baseline regression. Their purpose was to assess whether the direction of pairwise association differed from the conditional linear relationship, following the interpretive distinctions described by Schober et al., 2018. Two additional sensitivity analyses addressed individual observations and measurement construction. First, the regression was refitted 60 times, omitting one lineage in each fit. Each regression retained 59 observations, and coefficient ranges assessed changes in direction and magnitude. Second, the median of each technical triplicate replaced the corresponding maximum likelihood concentration estimate. Parental denominators were reconstructed using the same median rule before normalization and reestimation. This comparison retained all 60 lineages.

The analyses were exploratory and were not preregistered. Sensitivity procedures did not change eligibility rules or remove observations from the baseline sample. All three conditional estimates were retained regardless of their probabilities. Interpretation considered magnitude, confidence intervals, measurement scale and sensitivity rather than treating statistical significance as proof of biological importance or its absence as proof of no association. The supplied workbook preserves source measurements, lineage identifiers, parental benchmarks, calculation formulas and the variable dictionary. Equation 3 specifies the implemented inference procedure, while Tables 5 and 6 document the principal estimates and association diagnostics. Preserving these connections supports computational traceability, consistent with Sandve et al., 2013. The methodological contribution is the coordinated application of established procedures to distinguish conditional resistance associations from susceptibility changes relative to the parent, rather than a new estimator or causal identification design.

5. Findings:

This analysis examines how cefotaxime, cefazolin and ceftolozane/tazobactam resistance relate to gentamicin susceptibility across 60 evolved Escherichia coli lineages. The findings distinguish unadjusted associations from conditional regression estimates. Diagnostic and sensitivity analyses assess whether measurement choices, predictor overlap or individual observations alter the conclusions. The analytical sample contains 60 unique lineages assessed after passage ten of cefotaxime selection. All observations have complete measurements for the three predictors and gentamicin outcome. The parental strain provides normalization benchmarks and is excluded from the regression sample. Technical triplicates support the concentration estimates but do not increase the number of independent lineages. The observations represent one experimental endpoint from the dataset published by Nichol et al., 2019. Each analytical measure is the base two logarithm of the lineage minimum inhibitory

concentration divided by the corresponding parental concentration. Higher predictor values indicate greater beta lactam resistance. Higher gentamicin values indicate lower susceptibility. A negative regression coefficient therefore indicates that greater beta lactam resistance is associated with greater gentamicin susceptibility.

Table 4: Distribution of normalized resistance and gentamicin response

Measure	Valid Observations	Mean	Standard Deviation	Median	Minimum	Maximum
Cefotaxime resistance	60	5.567	2.902	4.000	2.000	10.000
Cefazolin resistance	60	3.717	0.825	4.000	2.000	5.000
Ceftolozane/tazobactam resistance	60	2.883	0.976	3.000	1.000	5.000
Gentamicin response	60	0.550	0.565	1.000	−1.000	2.000

Cefotaxime resistance exhibits the widest variation, spanning fourfold to 1,024-fold increases relative to the parental concentration. Cefazolin resistance ranges from fourfold to 32-fold, while ceftolozane/tazobactam resistance ranges from twofold to 32-fold. These distributions establish variation in every predictor without requiring a composite resistance index. Gentamicin response is concentrated at four measured levels. One lineage has a concentration below the parental estimate, 26 have the same estimate and 33 have higher estimates. These groups represent 1.7%, 43.3% and 55.0% of the sample, respectively. Thus, greater gentamicin susceptibility relative to the parent is uncommon, even though gentamicin response varies systematically across resistance profiles. A negative association across evolved lineages must therefore be distinguished from widespread collateral sensitivity relative to the ancestor. This distinction follows the separation between collateral sensitivity and cross resistance discussed by Pál et al., 2015. The joint regression identifies cefotaxime resistance as the clearest conditional correlate of gentamicin response. Holding cefazolin and ceftolozane/tazobactam resistance constant, a doubling of the cefotaxime concentration is associated with a 0.131 decrease in normalized gentamicin response. The 95% confidence interval extends from a decrease of 0.207 to a decrease of 0.056. On the concentration scale, the estimate corresponds to an approximately 8.7% lower fitted geometric mean gentamicin concentration, with an interval from 3.8% to 13.3% lower.

Table 5: Joint associations with normalized gentamicin response

Term	Coefficient	HC3 Standard Error	Lower 95% Confidence Limit	Upper 95% Confidence Limit	p Value
Cefotaxime resistance	−0.1314	0.0375	−0.2065	−0.0563	0.0009
Cefazolin resistance	−0.0375	0.0955	−0.2287	0.1537	0.6959
Ceftolozane / tazobactam resistance	0.0739	0.1055	−0.1373	0.2852	0.4862
Intercept	1.2077	0.3512	0.5042	1.9112	0.0011

The cefotaxime estimates answers RQ1 by identifying an inverse association with gentamicin concentration, equivalent to greater susceptibility across evolved lineages. This relationship does not establish that cefotaxime resistance causes gentamicin sensitivity. Different evolutionary pathways may jointly influence both phenotypes. Such contingency is consistent with the contrasting collateral outcomes documented by Barbosa et al., 2017. Cefazolin resistance shows a smaller negative conditional estimate. A doubling corresponds to an approximately 2.6% lower fitted geometric mean gentamicin concentration. However, its confidence interval permits changes ranging from 14.7% lower to 11.2% higher. RQ2 therefore yields an imprecise association whose direction is not resolved by the joint model. The estimate does not establish either an independent susceptibility advantage or the absence of a relationship.

Ceftolozane/tazobactam resistance has a positive conditional estimate. A doubling corresponds to an approximately 5.3% higher fitted geometric mean gentamicin concentration, but the confidence interval ranges from 9.1% lower to 21.9% higher. RQ3 therefore does not identify a precise conditional direction. The interval accommodates both greater and lower gentamicin susceptibility. The joint model accounts for 35.1% of the observed variation in normalized gentamicin response. Adjusted explained variation is 31.6%, and the residual standard error is 0.467 logarithmic units. These quantities describe fit within the observed sample and do not establish predictive performance in independent lineages.

Unadjusted rank correlations clarify why the conditional estimates require separate interpretation. Cefotaxime resistance has an inverse Spearman correlation with gentamicin response of −0.569. Ceftolozane/tazobactam resistance also has an inverse correlation of −0.419, whereas cefazolin resistance has a weaker correlation of −0.160. The ceftolozane/tazobactam relationship changes direction after conditioning on the other resistance measures, indicating that its unadjusted pattern does not translate into a distinct inverse association in the joint model. Rank correlations describe monotonic relationships and should not be interpreted as conditional or causal effects, consistent with Schober et al., 2018.

Table 6: Unadjusted rank associations and predictor overlap

Predictor	Spearman Correlation With Gentamicin Response	Approximate p Value	Variance Inflation Factor
Cefotaxime resistance	−0.569	< 0.0001	2.431
Cefazolin resistance	−0.160	0.2215	1.086
Ceftolozane / tazobactam resistance	−0.419	0.0009	2.555

The design matrix has full rank. Variance inflation factors indicate some shared variation between cefotaxime and ceftolozane / tazobactam resistance, but no exact linear redundancy. Consequently, the difference between unadjusted and conditional associations is substantively relevant rather than a reason to remove a predictor mechanically. Residual assessment identifies a departure from normality, with a Shapiro Wilk statistic of 0.944 and p = 0.0084. The outcome itself occupies only four logarithmic levels, which limits the continuous approximation underlying the linear model. A Breusch Pagan diagnostic gives a

statistic of 0.637 and p = 0.8879, providing little evidence of the variance pattern tested. This result does not establish constant variance. HC3 inference remains an approximate safeguard against unequal variance and leverage effects, rather than a correction for every specification problem, as explained by Mansournia et al., 2021.

Sensitivity analyses support the stability of coefficient direction while preserving uncertainty about magnitude. Across 60 regressions that each omitted one lineage, the cefotaxime coefficient ranged from −0.149 to −0.115. The cefazolin coefficient ranged from −0.083 to −0.005, and the ceftolozane/tazobactam coefficient ranged from 0.033 to 0.139. No individual omission reversed any coefficient's sign. The largest leverage value was 0.240 for lineage X44, but the omission analysis indicates that the cefotaxime direction was not determined by that observation alone. Replacing each maximum likelihood concentration estimate with the median of its three technical measurements produced identical analytical values for all four drugs and all 60 lineages. The regression estimates therefore remained unchanged under this alternative measurement construction. This agreement supports numerical consistency within the source measurements without establishing independent experimental replication. The evidence answers the three research questions asymmetrically. Cefotaxime resistance has a negative conditional association with gentamicin concentration that persists across the implemented sensitivity checks. Cefazolin and ceftolozane/tazobactam resistance yield imprecise conditional estimates. These findings describe differences among experimentally evolved lineages and do not establish clinical treatment effects or broadly transferable antibiotic combinations.

6. Discussion:

Greater resistance to a selecting antibiotic can coincide with greater susceptibility to another drug without producing widespread collateral sensitivity. This distinction is the central contribution of the analysis. Table 5 identifies an inverse conditional association between cefotaxime resistance and gentamicin concentration, while Table 4 shows that gentamicin concentrations generally remain at or above the parental benchmark. Equation 1 separates these two comparisons: variation among evolved lineages and change relative to their ancestor. The contribution therefore lies in distinguishing a relative susceptibility gradient from an ancestral susceptibility advantage. Nichol et al., 2019, established heterogeneous collateral responses in the source experiment; the present analysis characterizes how those responses relate to a jointly specified resistance profile. This refines interpretation of evolutionary tradeoffs because an inverse association alone does not establish a useful collateral sensitivity pair. The contrast between unadjusted and conditional estimates shows why resistance phenotypes require joint interpretation. Ceftolozane/tazobactam resistance has an inverse rank association with gentamicin response in Table 6, yet its coefficient becomes positive and imprecise in Table 5. Under Equation 2, its apparent susceptibility advantage is therefore not distinguishable from variation shared with the other resistance measures. This change concerns statistical conditioning rather than a demonstrated biological transmission pathway. Barbosa et al., 2017, showed that alternative evolutionary routes can produce contrasting collateral responses among populations selected with the same antibiotic. The present evidence adds a complementary analytical distinction: a relationship visible between two phenotypes may not remain independently informative when the broader resistance profile enters the model.

Cefotaxime provides the clearest conditional association, but the estimates do not establish biological dominance over the other resistance dimensions. Table 5 supports a negative cefotaxime coefficient, whereas the confidence intervals for cefazolin and ceftolozane/tazobactam accommodate both susceptibility gains and losses. Equation 2 identifies conditional associations rather than separable shares of an evolutionary effect. The theoretical implication is that membership in the same antibiotic class does not make resistance phenotypes interchangeable predictors of another drug response. Pál et al., 2015, explain that collateral effects depend on the physiological consequences of resistance adaptations. By retaining distinct observed measures, this analysis avoids treating a general resistance score as sufficient evidence about gentamicin susceptibility. The measurement distribution places a substantive boundary on the apparent opportunity for exploiting these associations. Only one lineage has a gentamicin concentration below the parental estimate, while most occupy unchanged or higher concentration categories. Consequently, the negative cefotaxime coefficient in Table 5 describes relative differences within an evolved population that remains predominantly unchanged or less susceptible than its ancestor. The stability of coefficient signs under individual omissions and the identical results obtained from technical medians support numerical consistency, but they do not convert this pattern into clinical effectiveness. Allen et al., 2021, demonstrated that assay environments can alter collateral responses. Together with the endpoint normalization in Equation 1, this evidence makes the reference strain and experimental conditions integral to interpretation rather than incidental features of measurement.

The international relevance concerns how laboratories interpret resistance profiles across experimental systems, rather than differences between national economies. Munck et al., 2014, examined resistance development under antibiotic combinations, whereas the estimates in Table 5 concern endpoint responses following cefotaxime selection. These designs answer different questions: a conditional association after selection does not establish that simultaneous treatment will constrain resistance. Equation 2 can therefore support laboratory prioritization of associations for further evaluation, but it cannot rank clinically effective combinations. The practical change is to assess both the conditional relationship and the ancestral benchmark before interpreting an inverse association as a therapeutic opportunity. This extends the analytical use of collateral response data while preserving the distinction between phenotypic association and experimentally demonstrated control of resistance evolution.

7. Conclusion and Implications:

Exploiting antibiotic resistance requires distinguishing relative susceptibility gains from genuine restoration of ancestral sensitivity. This analysis reveals that resistance profiles contain unequal information about collateral drug responses, while an inverse association among evolved lineages can coexist with predominantly unchanged or reduced susceptibility relative to their ancestor. The contribution is an empirical distinction between conditional associations within an evolved population and susceptibility advantages against a parental benchmark. This distinction refines evolutionary trade off reasoning by showing why resistance gradients cannot independently establish exploitable therapeutic opportunities. Joint interpretation also demonstrates that an apparent susceptibility advantage can disappear after accounting for shared phenotypic variation. For research managers, the findings support allocating experimental resources according to both conditional associations and ancestral comparisons, rather than prioritizing antibiotic pairs through isolated correlations. Laboratory workflows can operationalize this approach by

preserving reference measurements, documenting technical replication and evaluating overlapping resistance profiles before advancing candidates. Research funding and antimicrobial innovation policies can require these distinctions when assessing evidence for experimental treatment strategies. The broader contribution is a disciplined basis for interpreting collateral response data across laboratories and bacterial systems. Its potential social value lies in strengthening the evidence used to prioritize antimicrobial research, while keeping therapeutic decisions within demonstrated efficacy.

The experimental endpoint design provides a focused platform for longitudinal extensions that distinguish transient susceptibility patterns from persistent evolutionary constraints. Repeated measurements across selection passages could establish how associations emerge and change. Experiments involving additional strains and environmental conditions could assess transferability beyond the observed genetic background. Genetic reconstruction and physiological assays could test the mechanisms underlying the conditional relationships, while independently assigned exposure sequences could strengthen causal identification. Replication using additional susceptibility measurements and independent populations would assess measurement sensitivity and generalization. Subsequent treatment experiments could determine whether the observed associations translate into reproducible suppression of resistance evolution.

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