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ORIGINAL Article

Experimental Induction of Resistance in Escherichia coli to different antibiotic groups through repeated fixed drug exposure

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ABSTRACT

The overuse of antibiotics in healthcare has accelerated the emergence of antimicrobial resistance (AMR) and the development of multi drug-resistant "superbugs" worldwide. The study aimed to induce resistance in standard ATCC Escherichia coli strain by fixed concentration of different antimicrobial groups through repeated exposure in our institutional laboratory.

E. coli, ATCC 25922 strain which was initially susceptible to antibiotics was serially exposed to five groups of antibiotics (ampicillin, co-trimoxazole, tetracycline, gentamicin, and amoxicillin–clavulanate) over 60 daily passages. Antibiotic susceptibility testing (AST) was performed by Kirby–Bauer disk diffusion method on Müller-Hinton agar with a standardised inoculum (~10^6 CFU). On day 0, a Muller Hinton Agar (MHA) plate was inoculated with the broth and mentioned antibiotic disks were placed for zone diameter estimation. Thereafter, for each next passage, colonies from the edge of the inhibition zone for each drug were picked and inoculated on fresh MHA plates and were further rechallenged with the same antibiotic discs. Inhibition zone diameters were measured after overnight incubation at 37°C.

On day 0, E. coli was fully susceptible to all five antibiotics. After 60 serial passages, the E. coli population exposed to ampicillin developed resistance, with the inhibition zone decreasing from 19 mm initially to resistant zone of 6 mm.

This work provides insights into the mechanisms by which E. coli adapts to sustained antibiotic pressure and offers a basis for developing strategies to prevent the rise of multi drug-resistant bacteria.

Keywords
E. coli, Lab-induced resistance, Fixed dose, Antimicrobial Resistance, Antibiotics.

Abbreviations
Ampicillin (AMP); Amoxicillin–clavulanate (AMC); Antimicrobial resistance (AMR); Antibiotic susceptibility testing (AST); Carbapenem-resistant Enterobacteriaceae (CRE); Colony-forming units (CFU); Co-trimoxazole (COT); Escherichia Coli (E.Coli); Extensively drug-resistant (XDR); Gentamicin (GEN); Intermediate (I); Muller Hinton Agar (MHA); Multidrug-resistant (MDR); Resistant (R); Susceptible (S); Tetracycline (TE).

INTRODUCTION

Antimicrobial resistance has been called a "silent pandemic," a slow-burning catastrophe that often remains unnoticed until its consequences become dire[1]. Infections by drug-resistant bacteria are currently estimated to cause about 700,000 deaths globally per year, and this toll could rise to 10 million per year by 2050, if no effective action is taken[2]. The

economic impact is likewise alarming: the World Bank projects that uncontrolled AMR could slash up to 3.8% of global GDP by 2050, pushing millions into poverty[3]. Over seventy years ago, Alexander Fleming forewarned in his 1945 Nobel prize speech addressing that improper use of penicillin and exposing microbes to sub-lethal concentrations would inevitably lead to the selection of resistant organisms. Within a decade of penicillin's widespread use, *Staphylococcus aureus* became resistant by producing penicillinase enzyme. By the 1960s, certain *Klebsiella pneumoniae* and *Escherichia coli* isolates had already acquired resistance to multiple antibiotics. The following decades saw an accumulation of multidrug-resistant organisms (MDROs), with the 1980s–1990s marking the rise of resistance to many first-line drugs, and the early 2000s bringing the spread of carbapenem-resistant *Enterobacteriaceae* (CRE)[4].

The burden of AMR falls heaviest on low- and middle-income countries, where high infection rates, antibiotic overuse, and fewer resources for infection control facilitate the proliferation of resistant strains. In India, for example, a multicenter retrospective study found that hospital infections caused by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *E. coli*, *K. pneumoniae*, and *Acinetobacter baumannii* were associated with mortality rates 2–3 times higher than those of infections by susceptible strains[4]. In intensive care units, patients infected with MDR Gram-negative bacteria have reported in-hospital mortality rates of 20–38%, compared to around 10% in uninfected patients[5].

Several other factors drive the AMR crisis in regions like India, including the overprescription of antibiotics, easy over-the-counter access to antimicrobial drugs, environmental contamination (e.g. pharmaceutical and agricultural run-off), and widespread antibiotic use in livestock and poultry[6]. Bacteria have evolved many strategies to withstand antibiotic attack. Well-known resistance mechanisms include enzymatic drug inactivation (e.g. β-lactamase production), modification of the antibiotic's target site to reduce binding affinity, reduced permeability or active efflux of the drug to prevent it from reaching its target, and the use of alternative metabolic pathways to bypass the drug's effect[7]. Each course of improper or repeated antibiotic use applies selective pressure to bacterial populations, favoring the survival of resistant variants while susceptible bacteria are killed off. Over time, this process can make resistance genes and mutations more prevalent[8]. Most experimental studies of antibiotic resistance focus on scenarios where bacteria acquire the ability to grow under increasing concentrations of a drug (i.e. selecting for higher MICs) [9,10]. In contrast, our study examines the evolution of resistance under constant drug exposure simulating a clinical situation where a patient is treated repeatedly with the same antibiotic dose. By using an adaptive laboratory evolution approach[11], we tracked how a susceptible *E. coli* strain transitions to a resistant state when challenged daily with the same antibiotic. This approach sheds light on the subtle genetic and phenotypic changes that accumulate under sustained selective pressure, offering insights relevant to both the development of new antimicrobials and the prudent use of existing ones.

The purpose of the study was to induce resistance in vitro through repeated exposure of fixed dose of antibiotics and to find out with the number of serial passages needed for the same. Understanding this progression can help clarify the contribution of such repeated exposure to treatment failure in infections.

MATERIALS AND METHODS

This study was carried out in the institutional research laboratory, School of Medical Sciences and Research (Greater Noida, Uttar Pradesh, India) over a three-month period (Feb–Apr 2025).

Bacterial Strain and Culture Conditions

The test organism selected was *Escherichia coli* ATCC 25922, strain that was initially susceptible to used of antibiotics. The bacterium was cultured on Müller-Hinton Agar (MHA) and incubated at 37°C for 24 hours to ensure a fresh, actively growing culture for each experiment.

Antibiotics Tested

Five agents were selected from different antimicrobial groups for the induction experiment, representing different classes commonly used in clinical practice namely: ampicillin (AMP) 10 µg (a β-lactam penicillin), co-trimoxazole (COT) 25 µg (trimethoprim–sulfamethoxazole, a folate synthesis inhibitor combination), tetracycline (TE) 30 µg (a tetracycline class ribosomal inhibitor), gentamicin (GEN) 10 µg (an amino glycoside), and amoxicillin–

clavulanate (AMC) 20/10 µg (a penicillin β-lactam combined with a β-lactamase inhibitor). All antibiotic disks and concentrations were obtained from standard suppliers and used according to CLSI guidelines for disk diffusion[12].

Adaptive Exposure Procedure

We employed a serial passage (adaptive laboratory evolution) method to induce resistance. A first bacterial inoculum of approximately 1×10^6 colony-forming units per mL (CFU/mL) was prepared by diluting an overnight *E. coli* culture into sterile saline to match the 0.5 McFarland turbidity standard. For Day 0, this standardised inoculum was lawn-cultured onto an MHA plate. Five different antibiotic disks (AMP, COT, TE, GEN, AMC) were then placed on the agar surface, spaced ~20 mm apart to ensure distinct inhibition zones without overlapping. The plate was incubated at 37°C for 16–18 hours. After incubation, we measured the diameter of the inhibition zone around each disk in millimeters (mm), following CLSI disk diffusion method.

For Day 1 and onward, we started separate lineages for each antibiotic. From the Day 0 plate, a colony found at the edge of the inhibition zone for each antibiotic was identified (this represents bacteria that had the greatest ability to grow near the antibiotic). Using a sterile loop, that colony was picked and suspended into fresh saline to prepare a new ~10^6 CFU/mL inoculum. This suspension was then used to inoculate a new MHA plate containing only the same antibiotic disk corresponding to that lineage. In other words, starting on Day 1 each antibiotic had its own plate and subculture line. The plate was incubated overnight, and the zone of inhibition measured the next day, as before. This process (selecting a perimeter colony and transferring it to a new plate with the same antibiotic disk) was repeated daily for each antibiotic for a total of 60 serial passages (designated Day 1 through Day 60). We maintained a constant antibiotic disk potency and did not increase the antibiotic concentration at any point, thereby subjecting the bacteria to an unchanging level of drug concentration each day to simulate the condition in vivo where patient is exposed to similar dosage of antibiotics.

Monitoring and Quality check

The primary measure of emerging resistance was the change in disk diffusion inhibition zone diameter over successive passages. According to CLSI M100 standards, each antibiotic has defined breakpoints classifying the *E. coli* isolate as Susceptible (S), Intermediate (I), or Resistant (R) based on the zone size (**Table 1**). We recorded the zone diameter for each antibiotic on each day. A decrease in zone diameter, especially one crossing below the susceptible breakpoint, was interpreted as the development of reduced susceptibility or resistance. The experiment was repeated 60 times until a definite resistance phenotype (as defined by CLSI standards) was seen for any antibiotic.

To confirm the reproducibility of the resistance phenotype, we performed periodic confirmation tests. We took the evolved *E. coli* isolates (from each antibiotic lineage) from Day 15, Day 45, and Day 60 and re-assessed the zone diameter on a new MHA plate with the corresponding antibiotic disks. This served as a quality control to ensure that the observed changes in inhibition zones were fixed and were reproducible throughout the experiment. We then compared the inhibition zone from the repeat test to the original measurement for that day.

Data Analysis

Zone diameters were tabulated for each antibiotic across all 60 days. We focused on the change from Day 0 (baseline) to Day 60 for each drug, and noted on which day, if any, the zone fell into the resistant range. The results are summarised in tables and figures, with *E. coli* ATCC 25922 reference susceptibility ranges serving as controls.

RESULTS

Initial Susceptibility (Day 0)

The *E. coli* ATCC 25922 strain was confirmed to be susceptible to all five antibiotics at the start of the experiment. On Day 0, clear inhibition zones were observed around each antibiotic disk, with diameters within the susceptible range defined by CLSI. **Table 1** shows the measured zone diameters on Day 0 for each drug alongside the CLSI breakpoints. For example, ampicillin produced a 19 mm zone (≥17 mm indicates susceptibility), gentamicin a 22 mm zone (≥18 mm is susceptible), tetracycline 21 mm, cotrimoxazole 29 mm, and amoxicillin–clavulanate 19 mm. These values all fell in the susceptible category (**Table 1**).

Development of Resistance over 60 Passages

Over the course of the serial exposures, we observed a progressive decline in inhibition zone size for all

Table 1. *Initial and final zone diameter (millimetres) with respect to CLSI guidelines*. Summary of the initial (Day 0) and final (Day 60) zone diameters for each antibiotic, along with the CLSI breakpoint criteria for susceptibility (S), intermediate (I), and resistance (R).

Drug	D_0	D_{60}	CLSI		
			S	I	R
Ampicillin	19	6	≥17	14-16	≤13
Amoxicillin–Clavulanate	19	17	≥18	14-17	≤13
Gentamicin	22	21	≥18	15-17	≤14
Tetracycline	21	20	≥11	12-14	≤15
Cotrimoxazole	29	21	≥10	11-15	≤16

(Day 0 (D_0), Day 60 (D_{60}), S- Susceptible, R- Resistance, I- Intermediate)

antibiotics, though to varying extents (**Table 2**). Notably, the *ampicillin-exposed lineage* showed the most dramatic change. The zone of inhibition for ampicillin steadily diminished with each passing week of daily exposure. By approximately the 30th–40th passage, the ampicillin zone had shrunk into the intermediate range, and by the end of the 60th passage it measured only 6 mm. According to CLSI criteria, *E. coli* is categorised as ampicillin-resistant if the zone is ≤13 mm; thus, our strain unequivocally became ampicillin-resistant by the conclusion of the experiment. In contrast, none of the other antibiotic lineages achieved the established resistance breakpoint within 60 days, although all showed a measurable reduction in susceptibility.

Table 2. *Inhibitory zone diameters* (mm) for *E. coli* over 60 serial passages under constant exposure to each antibiotic.

No	AMP	AMC	GE	TE	COT
1	19	19	22	21	29
2	20	19	25	24	28
3	20	19	25	24	26
4	20	19	25	24	26
5	20	20	24	25	29
6	19	20	24	24	26
7	20	21	25	24	26
8	20	20	24	24	26
9	20	20	25	25	26
10	20	21	25	24	27
11	20	20	25	24	26
12	20	21	24	24	26
13	19	16	25	22	27
14	20	20	24	22	26
15	20	18	21	26	25
16	20	18	26	26	30
17	20	17	30	26	26
18	20	19	23	25	30
19	18	19	23	27	28
20	16	18	23	25	29
21	16	18	24	23	27
22	18	18	25	23	27
23	18	16	23	23	27
24	17	18	24	23	25
25	16	18	24	21	24
26	18	18	26	21	28
27	19	16	23	24	26
28	19	12	23	27	28
29	17	12	23	25	25
30	17	12	24	25	25
31	16	12	22	25	24
32	15	17	22	19	28
33	15	17	24	20	26
34	14	15	24	20	26
35	14	19	21	20	26
36	13	17	21	19	26
37	13	17	21	21	23
38	11	16	24	22	27
39	10	15	23	23	30
40	10	15	21	23	28
41	10	13	21	21	29
42	9	14	21	20	27
43	9	16	24	20	25
44	9	16	23	21	25
45	8	16	20	20	22
46	8	18	22	22	23
47	8	16	20	20	22
48	8	18	20	22	24
49	8	18	30	22	24
50	8	18	23	21	23
51	8	18	21	23	23
52	8	17	21	21	23
53	8	16	21	21	23
54	8	18	23	20	22
55	6	17	22	20	21
56	6	18	22	21	22
57	6	18	22	21	21
58	6	17	21	21	22
59	6	17	21	20	21
60	6	17	21	20	21

- **Amoxicillin–Clavulanate (AMC):** Initial zone 19 mm (susceptible ≥18 mm). By Day 60 the zone was 17 mm, which straddles the borderline between susceptible and intermediate. While not officially "resistant" (CLSI resistant ≤13 mm for AMC), this decrease indicates reduced susceptibility. The amoxicillin–clavulanate lineage thus showed a slight waning of efficacy, but the bacteria remained largely inhibited by the drug (**Figure 1**).

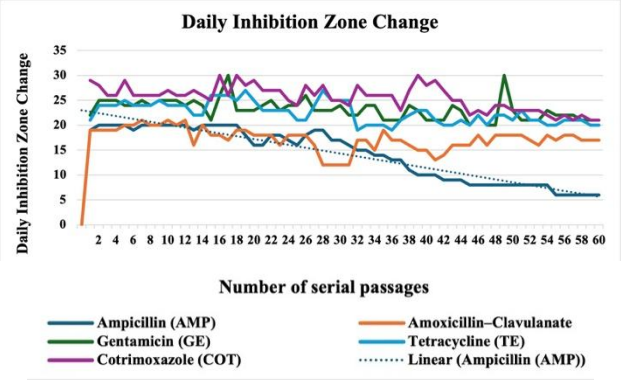

Figure 1. *Daily Inhibitory zone diameter* (mm) for E. coli over 60 serial passages

- **Gentamicin (GEN):** Initial zone 22 mm (susceptible ≥18 mm) The Day 60 zone was 21 mm, still well within the susceptible range. The decrease was minimal, suggesting that resistance to gentamicin did not emerge under the conditions and timeframe of our experiment.
- **Tetracycline (TE):** Initial zone 21 mm. By Day 60 this was 20 mm. According to CLSI, susceptible *E. coli* to tetracycline is typically ≥15 mm, so a 20 mm zone remains susceptible. The drop in zone size was modest, indicating no major resistance development to tetracycline in 60 passages.
- **Co-trimoxazole (COT):** Initial zone 29 mm. This decreased to 21 mm at Day 60. Cotrimoxazole susceptibility is defined by a zone ≥16 mm, so 21 mm is still comfortably in the susceptible range. However, a reduction of 8 mm over 60 days suggests some adaptive change, even if not enough to cause clinical resistance.

By the end of the 60-day evolution experiment, *E. coli* exposed to ampicillin met the definition of resistance, whereas for the other antibiotics the organism remained classified as susceptible (AMC being on the cusp of intermediate).

Table 2 details the daily course of inhibition zone changes for each antibiotic. In brief, the ampicillin inhibition zone showed a steady downward trend: from 19 mm on Day 0 to ~13–14 mm by Day 35, and into single digits by Day 50–60. The cotrimoxazole and tetracycline zones also shrank appreciably (by 8 mm and 1 mm respectively by Day 60), though still above resistance cutoffs. Gentamicin and amoxicillin–clavulanate exhibited relatively stable zones with only minor declines (1–2 mm decrease over 60 days).

Confirmation of Resistance Phenotype: To ensure that the observed reduction in ampicillin susceptibility was not a transient stress response, we performed repeat AST on the evolved ampicillin-resistant strain after 15, 45, and 60 passages (**Table 3**). The re-testing produced inhibition zones virtually identical to those obtained during the evolution experiment (for example, at passage 15 the original zone was 20 mm vs. 19 mm on repeat; at passage 45 it was 8 mm vs. 8 mm on repeat; at passage 60 it remained 6 mm on both tests). This consistency confirms that the resistance trait was stably inherited in the bacterial population and that our serial passage method reliably induced genetic changes rather than temporary adaptation. No reversion or loss of resistance was observed in the absence of antibiotic (though our protocol maintained continuous exposure, so reversal was not explicitly tested).

Table 3. Verification of ampicillin resistance via repeat disk diffusion tests

Serial subculture	Initial inhibitory zone (mm)	Inhibitory zone on repeat culture (mm)
15	20	19
45	8	8
60	6	6

The close agreement between original and repeat tests at days 15, 45, and 60 indicates that the reduced susceptibility to ampicillin was a stable characteristic of the evolved strain.

DISCUSSION

The rising burden of antibiotic resistance among Gram-negative bacteria, particularly *Escherichia coli*, poses a critical threat to global health. These organisms are often implicated in hospital-acquired infections and display remarkable adaptability to antimicrobial pressure. The challenge is compounded by the fact that bacteria do not follow a uniform or predictable path to resistance; they may acquire or upregulate a range of mechanisms, enzymatic degradation, target modification, decreased permeability, or efflux, that are influenced by both drug properties and environmental conditions.

In this study, we observed that repeated, fixed concentration exposure to antibiotics can possibly promote resistance in *E. coli* over time.

Despite the use of fixed drug concentrations, the bacterial population exposed to ampicillin developed high-level resistance by the 60th passage, while remaining susceptible to other antibiotics tested (**Table 1**). This finding aligns with reports by Toprak et al. (2012), who used an automated morbidostat to show that *E. coli* can rapidly evolve resistance under sustained, sublethal antibiotic pressure[13]. However, unlike adaptive systems that modulate drug levels in real time, our fixed-dose regimen better mirrors clinical settings where patients may receive standard, prolonged courses of antibiotics. Also, the zone reductions in our study were not steady as we found contrary zone increase from 16 mm (day 25) to18 mm (day26) in ampicillin which however decreased further to 15 (day32) (**Table 1**). The reason possibly could be presence of many colonies in near zone of inhibition and each among them could have different pace of induction.

Our results augment the notion that resistance can emerge even in the absence of increasing selective pressure, reinforcing findings by Wistrand-Yuen et al. (2018), who demonstrated that even minimal antibiotic concentrations can drive the selection of multidrug-resistant *E. coli* over time[14]. Notably, the resistance in our study was antibiotic-specific: while ampicillin resistance developed rapidly, other agents including gentamicin, tetracycline, co-trimoxazole, and amoxicillin–clavulanate did not reach full resistance within 60 passages, though some showed early signs of tolerance.

This selective resistance development may be attributed to the diversity and accessibility of resistance pathways. Ampicillin, a β-lactam antibiotic, is particularly vulnerable to a wide array of bacterial defences. These include β-lactamase production, porin channel modifications, PBP (penicillin-binding protein) mutations, and upregulation of efflux pumps, mechanisms well-documented in both clinical and laboratory strains of *E. coli*[15,16]. Our study did not specifically identify which of these mechanisms arose, but based on previous research, it is likely that endogenous β-lactamase upregulation or porin loss mutations played a role, as seen in adaptive evolution studies by Melnyk Ahet al. (2011)[17] .

In contrast, gentamicin and tetracycline require more complex resistance strategies, such as aminoglycoside-modifying enzymes or ribosomal protection proteins, which often incur higher fitness costs and may be slower to evolve under constant pressure[18]. Cotrimoxazole resistance typically involves dihydrofolate reductase mutations or metabolic bypasses, and although these have been observed in clinical isolates, their evolution under stable in vitro conditions may demand longer durations. Furthermore, the presence of clavulanate, a β-lactamase inhibitor in amoxicillin clavulanate, likely delayed resistance emergence by blocking a common escape route for *E. coli*, the enzymatic degradation of β-lactams.

Although only ampicillin resistance fully emerged within 60 days, we noted a general trend of declining susceptibility across the other antibiotics, suggesting that resistance development is a multi-phase process. Our findings support the hypothesis that the early stages of adaptation may occur silently, with resistant mutants gradually accumulating before crossing clinical breakpoints. Similar progressive trends were reported by Baym et al. (2016) using the "mega-plate" evolutionary experiment, which visualized resistance expansion across spatial antibiotic gradients[19]. While our study lacked spatial variation, the temporal dynamics revealed a comparable pattern of stepwise resistance emergence.

Clinically, these findings serve as a warning against prolonged or repeated use of the same antibiotic, even at standard doses. Repeated exposure enables bacteria to adapt incrementally, eventually culminating in full resistance. Cycling antibiotics or combining different drug classes could mitigate this risk, a principle emphasised in antibiotic stewardship programs worldwide.

From a research perspective, our use of serial passaging under fixed-dose antibiotic exposure provides a straightforward yet powerful model to study evolutionary resistance and could help in inducing resistance to different antibiotic groups. Future work incorporating genomic and transcriptomic analysis at defined time points (e.g., every 10 passages) could elucidate the precise sequence of mutations or regulatory shifts driving resistance. This approach has been effectively used in earlier studies to detect early molecular events, such as marA over-expression or loss-of-function mutations in outer membrane porins, which predispose *E. coli* to resistance development[20].

LIMITATIONS

It is important to acknowledge some limitations of our study. First, while we observed the phenotypic

emergence of resistance, we did not characterize the specific genetic mutations or molecular mechanisms underlying this change. As a result, we cannot say definitively which resistance mechanism (or combination) was responsible for the ampicillin resistance in our evolved strain. The study could be further extended to find out presence of gene mutation by sequencing of day 60 passage and comparing it with first passage at day 0. Second, our experiment was limited to one strain of *E. coli* and a short duration (60 days); different bacterial strains or species, or a longer experimental period, might yield more resistance outcomes. Third, the in vitro conditions cannot fully replicate the complexity of infections in living hosts factors like drug pharmacokinetics, host immune responses, and microbial interactions could not be assessed in our model. Therefore, while the qualitative finding of resistance development is highly relevant, the exact time limit and dynamics might differ in clinical scenarios.

Despite these limitations, our study contributes to a growing body of evidence that evolution of resistance is a foreseeable consequence of repeated antibiotic exposure and on the other hand it also suggested inapplicable method to induce resistance.

CONCLUSION

In summary, we tried to demonstrate that *E. coli* can evolve from a fully susceptible state to a resistant state for a β-lactam antibiotic (ampicillin) through daily serial exposure to the drug at a constant dose. This laboratory model mimics the selective pressure exerted by repetitive fixed dose antibiotic use and highlights how quickly resistance can appear even without escalating doses. The experiment underlines the importance of understanding the mechanisms of resistance development so that we can better prevent and counteract them. Our findings reinforce several key principles for combating AMR: (1) use Tablrantibiotics judiciously and only when necessary, (2) ensure adequate dosing and treatment duration to eradicate pathogens (thereby minimizing the survival of partially resistant cells), and (3) rotate or combine therapies when appropriate to avoid relentless pressure in a single direction. Continued research into the evolutionary pathways of resistance will inform the development of new antimicrobial agents designed to bypass or overcome common resistance mechanisms.

A multi-pronged approach is needed to address AMR. This includes antibiotic stewardship to slow the emergence of resistance, innovation in drug development (including adjunct therapies that suppress resistance mechanisms, such as β-lactamase inhibitors or efflux pump inhibitors), and public health interventions to reduce the spread of resistant strains. By applying the lessons learned from studies like ours, we can strategize to prolong the efficacy of existing antibiotics and ensure that future generations do not face untreatable bacterial infections.

Conflict of Interest

The authors declare no conflict of interest.

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Authors' contribution

LG screened through data and prepared the initial draft. LG and MS were involved in conceptualization, administration, interpretation of data and DK performed substantial revisions to the final draft. All authors have read and approved the final manuscript.

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