Assessing the efficacy of therapeutically promising combination of polymyxin B and triclosan against colistin resistant *Klebsiella pneumoniae*

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Abstract

Antimicrobial resistance (AMR) is one of the greatest public health challenges of current time and it is majorly associated with Gram-negative bacterial infections. Among these infections, *Klebsiella pneumoniae* is the most notorious bacterial pathogen in the context of AMR. The inefficiency of currently available antibacterial drugs is becoming common in hospitalized patients and clinicians feel hopeless during treatment. Eventually, clinicians explore old regimes such as colistin to treat these multidrug resistance infections, however, unfortunately, the resistance towards these last-resort drugs is also emerging rapidly. In this study, we examined the occurrence of colistin resistance in *Klebsiella pneumoniae* concerning its mechanism of resistance, chronology, and geographical distribution. We found that resistance, towards these last-resort drugs, colistin and polymyxin B, is emerging rapidly with evolving multiple mechanisms of resistance. Although various attempts are being made to find a solution for this crisis, a combination therapy comprising approved antibacterial drugs may be one of the most suitable strategies to fight against AMR in the current time-ticking situation. Therefore, we evaluated the efficacy of a therapeutically viable combination of polymyxin B and triclosan using a set of standard antibacterial assays for *in vitro* preclinical efficacy. We found that the combination is highly effective in a colistin-resistant clinical strain of *Klebsiella pneumoniae* as triclosan sensitizes the pathogen towards polymyxin B. Furthermore, the results of additional complementary experiments such as time-kill kinetics, biofilm inhibition, frequency of resistance, cell viability, and cytotoxicity assays show very encouraging results for the feasibility and validation of this combination. Finally, we examined the presence of mutations in field isolates of *Klebsiella pneumoniae* FabI enzyme as it has been hypothesized previously that triclosan may cause mutation in its binding target, FabI. Interestingly, we could not detect any mutation in the triclosan binding site of FabI in the genome of field isolates. Altogether, this study provides an overview of the current situation on colistin resistance, a promising solution for colistin resistance comprising a combination of two already being used antibacterial ingredients based on *in vitro* preclinical efficacy studies.

Keywords

Colistin resistance, *Klebsiella pneumoniae,* Antibacterial drug discovery, Combination therapy, Polymyxin B, Triclosan

1. Introduction

Antimicrobial resistance (AMR) has emerged as a critical global health issue and it poses a serious threat to healthcare systems worldwide, becoming one of the leading causes of mortality in the 21st century (Antimicrobial Resistance Collaborators, 2022; The Lancet, 2022). Various studies indicate that drug-resistant bacterial pathogens are responsible for approximately 700,000 deaths globally each year (Watkins and Bonomo, 2016). Statistical predictions paint a concerning picture, with estimations suggesting this number could rise to 10 million by 2050. The economic toll could be equally devastating, with potential losses reaching $100 trillion, highlighting the urgent need for global attention (de Kraker et al., 2016; Price, 2016).

Klebsiella pneumoniae, infamous for multidrug resistance, is a leading cause of nosocomial infections, including pneumonia, urinary tract infections, sepsis, and meningitis (Podschun and Ullmann, 1998). The World Health Organization (WHO) has identified this pathogen as a major concern for AMR due to its high rate of resistance against commonly used antibiotics for *K. pneumoniae* infections, such as penicillin, quinolones, and cephalosporins (Chen et al., 2014; Henson et al., 2017; Rawat and Nair, 2010). The emergence of multidrug-resistant (MDR) strains, including extended-spectrum beta-lactamase-producing (ESBL), carbapenem-resistant *K. pneumoniae* (CRKP), throw an urgent public health challenge (Gupta et al., 2011; Pitout and Laupland, 2008; Van Duin et al., 2013).

Polymyxins, notably colistin, and polymyxin B, have grabbed attention recently due to their effectiveness as potential drugs in the fight against MDR Gram-negative pathogens (Nation et al., 2014). These cyclic peptide antibiotics, polymyxin B and colistin, differing by a single amino acid, were initially utilized in the 1950s to treat infections caused by Gram-negative bacteria (Li et al., 2005). However, the alarming rise of MDR gram-negative pathogens resistant to aminoglycosides, fluoroquinolones, and β-lactams, including carbapenems, has driven a reconsideration of polymyxins as a “last-resort” therapeutic option for critically ill patients (Bialvaei and Samadi Kafil, 2015). In clinical uses, polymyxin B is directly administered in its active form, whereas colistin is employed as a prodrug, colistimethate sodium (CMS) (Tran et al., 2016).

The widespread use of colistin and polymyxin B has driven the emergence of resistance in *K. pneumoniae*. The first reported case of colistin-resistant *K. pneumoniae* was in May 2004, though the precise molecular mechanism of resistance was not fully understood at that time

(Antoniadou et al., 2007). Later on, it was found that colistin resistant in Gram-negative bacterial pathogens is associated with multiple mechanisms (Aghapour et al., 2019). In 2016, in a breakthrough report, a colistin resistant gene, "mobilized colistin resistance (*mcr-1*) discovered (Liu et al., 2016). Within a year, *mcr-1* gene is detected across the globe including Asia, Europe, North America, and South America (Skov and Monnet, 2016; Zhu et al., 2021). In 2017, WHO designated colistin resistance as a priority for the antibacterial development to treat colistin resistant infections (WHO, 2017).

mcr-1 gene produces lipid A phosphoethanolamine transferase which catalyse transfer of a phosphoethanolamine group to lipid A subsequently, altered lipid A lost affinity towards colistin and related polymyxins resulting in resistance in bacterial pathogens (Hinchliffe et al., 2017). The emergence of plasmid-mediated *mcr-1* resistance is particularly alarming, as it poses a significant risk of rapid dissemination of colistin resistance among *K. pneumoniae* and other bacterial species also (Bedenić et al., 2018; Granata and Petrosillo, 2017). Furthermore, it is concerning that higher mortality rates have been associated with colistin-resistant *K. pneumoniae* infections compared to susceptible strains, underscoring the urgent need for effective antimicrobial stewardship and novel therapeutic strategies (Rojas et al., 2017).

Although new classes of antibacterial drugs are in the pipeline, approval of these new drugs against novel targets in Gram-negative pathogens remains a significant challenge (Andrei et al., 2019). Furthermore, bacterial pathogens tend to develop resistance to antibiotics introduced in clinical settings, especially those that rely solely on antibacterial mechanisms, often within a very short period of time, sometimes in just months or years (Walsh, 2000). Consequently, exploring novel approaches, such as combination therapy, is showing promising results in combating bacterial AMR and enhancing therapeutic strategies for treating MDR infections (Tyers and Wright, 2019; Worthington and Melander, 2013). There are generally three types of combination therapy approaches: antibiotic-antibiotic, non-antibiotic with non-antibiotic, and non-antibiotic with antibiotic. Among these, several antibiotic-antibiotic combinations, such as Ceftazidime-Avibactam, Meropenem-Vaborbactam, Ceftolozane-Tazobactam, etc are approved previously (Salter 1982). Currently, non-antibiotic with non-antibiotic combinations is underexplored, especially regarding their effectiveness within the complex fluid environments of the human body (Liu et al., 2021). Therefore, combinations involving a non-antibiotic with an antibiotic are emerging as the most promising strategy for addressing AMR (Worthington and Melander, 2013). Non-antibiotic compounds can significantly enhance the efficacy of antibiotics when used in combination or act as potentiators.

Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol: TCS], a lipid-soluble synthetic biocide, was introduced in 1972 as a broad-spectrum antimicrobial agent within the healthcare industry. Since then, it has been widely used across North America, Asia, and Europe in various personal care products such as toothpaste, shampoo, detergent, mouthwash, soap, cosmetics, and deodorants, as well as in veterinary, household, and industrial products (Russell, 2004). Triclosan primarily targets the bacterial fatty acid synthesis (FAS II) pathway by inhibiting the activity of enoyl acyl-carrier protein reductase (ENR) (Levy et al., 1999; McMurry et al., 1998).

In this study, we examined the prevalence of the *mcr-1* gene in *K. pneumoniae* strains resistant to colistin. We analyzed 40,506 whole-genome sequences of *K. pneumoniae* available in the Pathogenwatch database, tracking the emergence of colistin resistance from 2009 to 2023. Our findings revealed that, following its detection in 2016, *mcr-1* prevalence has been increasing rapidly, along with an overall rise in colistin resistance. However, cases with *mcr-1* positivity remain lower than those exhibiting colistin resistance alone. Geographical distribution of resistance showed that Europe, North America, and Asia are the most affected continents, with Europe leading at 948 reported cases. In search of a solution for colistin resistance, we investigated a combination of polymyxin B and triclosan as promising antibacterial therapy using various efficacy assays. Preliminary resistance profiling and strain characterization were carried out on a colistin-resistant clinical strain of *K. pneumoniae*. Using a checkerboard assay, we demonstrated the synergistic efficacy of triclosan combined with polymyxin B in inhibiting the colistin-resistant clinical strain of *K. pneumoniae*. This combination reduced the minimum inhibitory concentration (MIC) of polymyxin B by 16-fold and triclosan by over 200-fold in the colistin-resistant strain, resulting in an excellent fractional inhibitory concentration (FIC) index of 0.066. Furthermore, Synergy Finder analysis showed a strong zero-interaction potency score of 27.78 for the combination. Biofilm assays further indicated that this combination fully inhibits biofilm formation. Cytotoxicity testing on the RAW 264.7 cell line showed that the cytotoxic effect was nearly null, further supporting the safety profile of the combination. Although triclosan is suspected to develop resistance through mutations in the active site residues of FabI, variant-calling analysis of the 36,000 *K. pneumoniae* isolates revealed no significant mutations in the binding site residues encoding sequence of *fabI* gene, despite the extensive use of triclosan in cosmetic products.

2. Results

2.1. *mcr-1* gene is not solely responsible for colistin resistance in *Klebsiella pneumoniae*

The analysis of 40,506 *K. pneumoniae* whole-genome data from 2009 to 2023, obtained from the publicly available, Pathogenwatch database, reveals a significant and alarming increase in colistin resistance over time (Fig 1). Resistance rates, which ranged between 4% and 8% in earlier years, escalated sharply to over 12% by 2023, indicating an upward trend that poses a serious challenge to the efficacy of colistin as a last-resort treatment. The plasmid-mediated *mcr-1* gene, a key contributor to colistin resistance, was first detected in 2016 in isolated cases. Since then, its prevalence has gradually increased, peaking in 2023 at less than 0.0004%. However, the significant disparity between the total number of colistin-resistant cases (2597) and *mcr-1* positive cases (88) strongly suggests that alternative resistance mechanisms, such as chromosomal mutations or non-*mcr-1* plasmid-mediated pathways, play a predominant role in driving colistin resistance in *K. pneumoniae*. These findings underscore the critical need for comprehensive genomic surveillance to monitor the emergence and dissemination of diverse resistance mechanisms and to guide effective containment strategies against MDR pathogens.

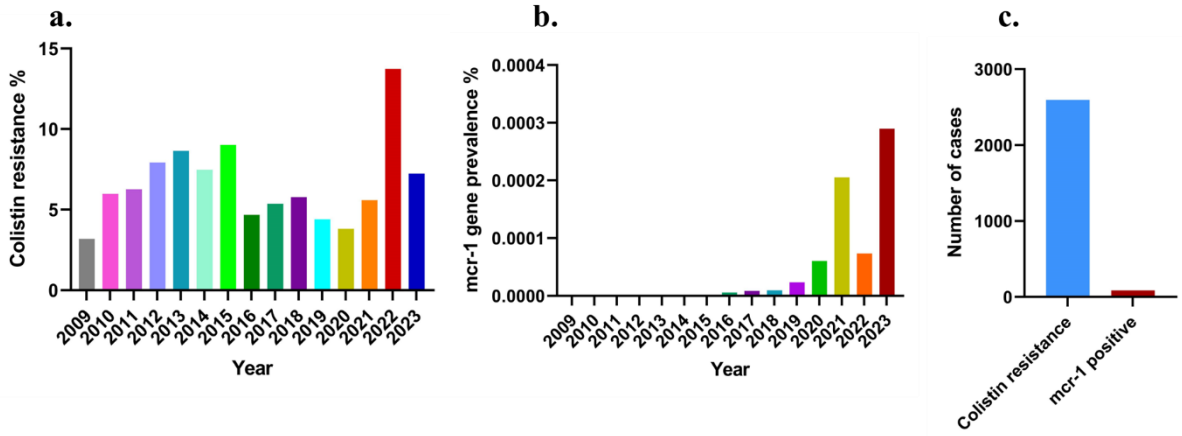

Fig 1: The prevalence of colistin resistance and the emergence of the *mcr-1* gene in *K. pneumoniae* have increased significantly between 2009 and 2023, (a.) The proportion of *K. pneumoniae* isolates resistant to colistin rose from about 5% in 2009 to over 15% by 2023, (b.) The *mcr-1* gene, a primary resistance gene in colistin resistant pathogens, was not detected in prior 2009. The first *mcr-1* positive strain was detected in 2016, and its prevalence climbed to over 0.003% by 2023, (c.) The total number of colistin-resistant cases grew from a few hundred in 2009 to more than 2,500 in 2023. Among these, *mcr-1* positive cases increased from nearly zero to 88 by 2023.

2.2. Global prevalence of colistin resistance in *Klebsiella pneumoniae*

The map illustrates the global distribution of *K. pneumoniae* sequences exhibiting colistin resistance, retrieved from the Pathogenwatch database for the period 2009 to 2023 (Fig 2). Europe reports the highest number of cases, with 948 sequences, indicating a significant burden of colistin-resistant *K. pneumoniae* in this region. North America follows with 353 cases, reflecting a substantial prevalence, while Asia reports 293 cases, highlighting its role as a significant region for resistance emergence. Africa accounts for 100 cases, and South America and Australia report lower numbers, with 42 and 27 cases, respectively. These findings emphasize the global dissemination of colistin resistance in *K. pneumoniae*, with particularly high prevalence in Europe and North America. This data underscores the critical need for enhanced surveillance systems and region-specific strategies to mitigate the spread of resistance and address this pressing public health challenge.

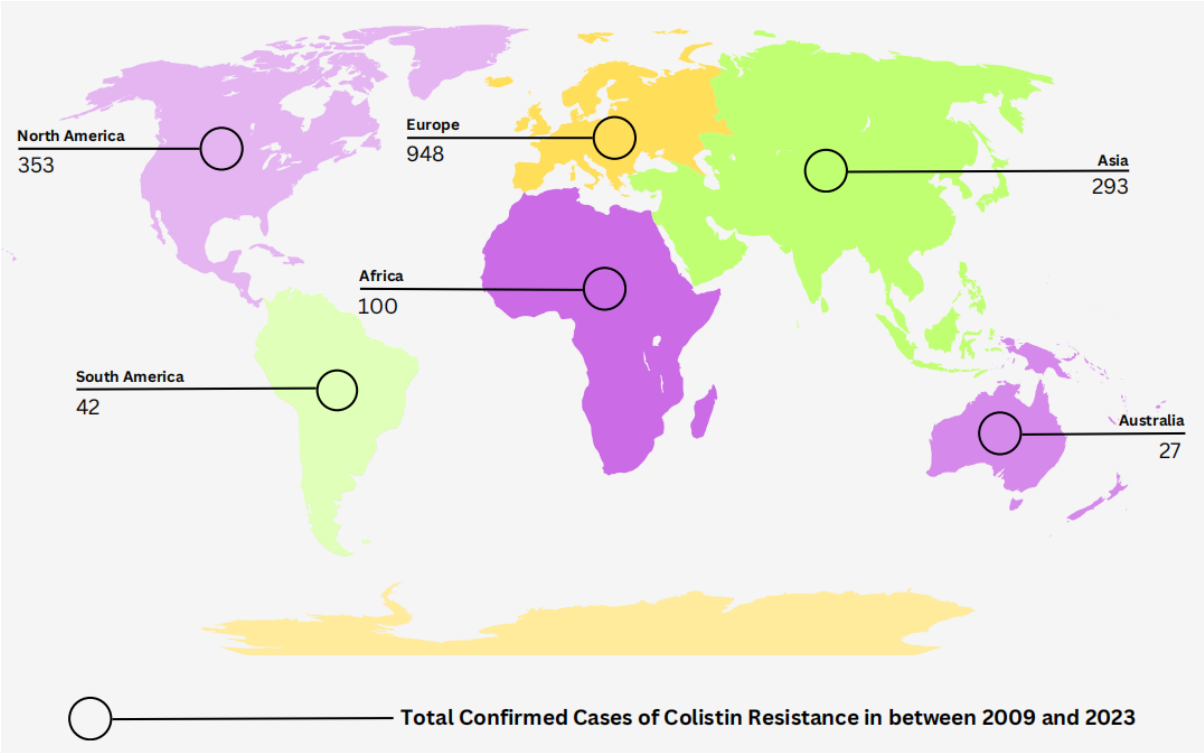

Fig 2: Global distribution of *K. pneumoniae* sequences exhibiting colistin resistance, retrieved from the Pathogenwatch database for the period 2009–2023. The map highlights the regional variability in resistance cases, with Europe showing the highest number of confirmed

sequences (948), followed by North America (353) and Asia (293). Africa reports 100 cases, while South America and Australia have significantly fewer cases, with 42 and 27, respectively.

2.3. Triclosan synergistically potentiates polymyxin B activity in colistin resistant *Klebsiella pneumoniae*

The combination of polymyxin B and triclosan demonstrates a strong synergistic effect against colistin -resistant *K. pneumoniae*, as confirmed through a series of experiments and analyses. The checkerboard broth microdilution assay showed a substantial reduction in the minimum inhibitory concentrations (MICs) of both drugs when used in combination. Specifically, the MIC of polymyxin B decreased from 16 µg/mL to 1 µg/mL, representing a 93.75% reduction, while the MIC of triclosan was reduced from 0.5 µg/mL to 0.0019 µg/mL, a significant 99.62% reduction, indicating enhanced antimicrobial activity (Table 1). The results of this assay were used to calculate the Fractional Inhibitory Concentration (FIC) index, which was found to be 0.0663, well below the threshold of 0.5, confirming a strong synergistic interaction (Fig 3). Subsequently, the synergy was quantified using the SynergyFinder application (version 3.0), which generated a ZIP synergy score of 27.78, further validating the pronounced synergy between the two antibiotics. Visual analyses, including a 2D synergy map and 3D δ-score plots, highlighted regions of optimal synergy across various concentration combinations, while the inhibition heatmap confirmed near-complete microbial inhibition (100%) in specific combinations (Fig 4). Collectively, these findings provide robust evidence that triclosan and polymyxin B, when used together, exhibit exceptional synergistic activity, making this combination a promising strategy against resistant *K. pneumoniae*.

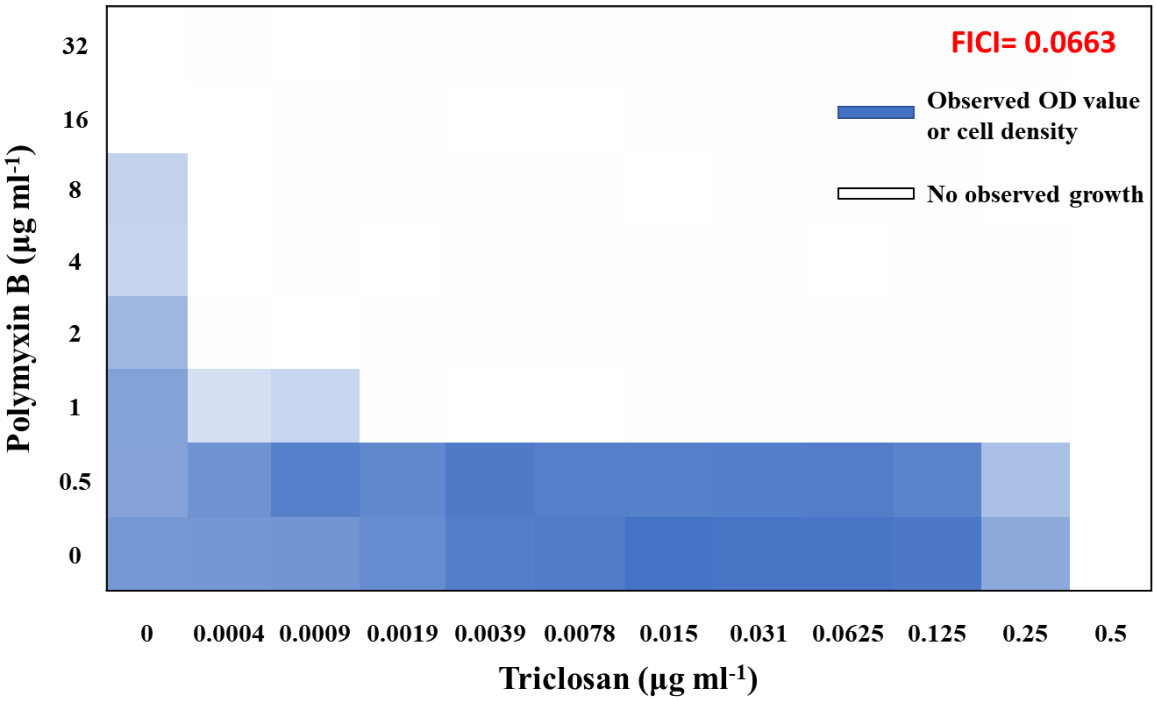

Fig 3: Chequerboard broth microdilution assays of polymyxin B and triclosan against colistin resistant *K. pneumoniae*. The figure displays a gradient of cell densities represented by different shades: dark regions indicate higher OD values or cell densities; lighter regions indicate lower cell densities and white regions indicate no observed growth. The chequerboard and FICI data shown represent results from at least three independent experiments.

Table 1: Percentage reduction of MIC of colistin resistant *K. pneumoniae* with combination therapy of triclosan and polymyxin B

Antimicrobial	MIC (µg/ml)			FIC		Effect
	Alone	In combination	Percentage reduction	FIC	FIC index	
Polymyxin B	16	1	93.75%	0.0625	0.0663	Synergistic
Triclosan	0.5	0.0019	99.62%	0.0038		

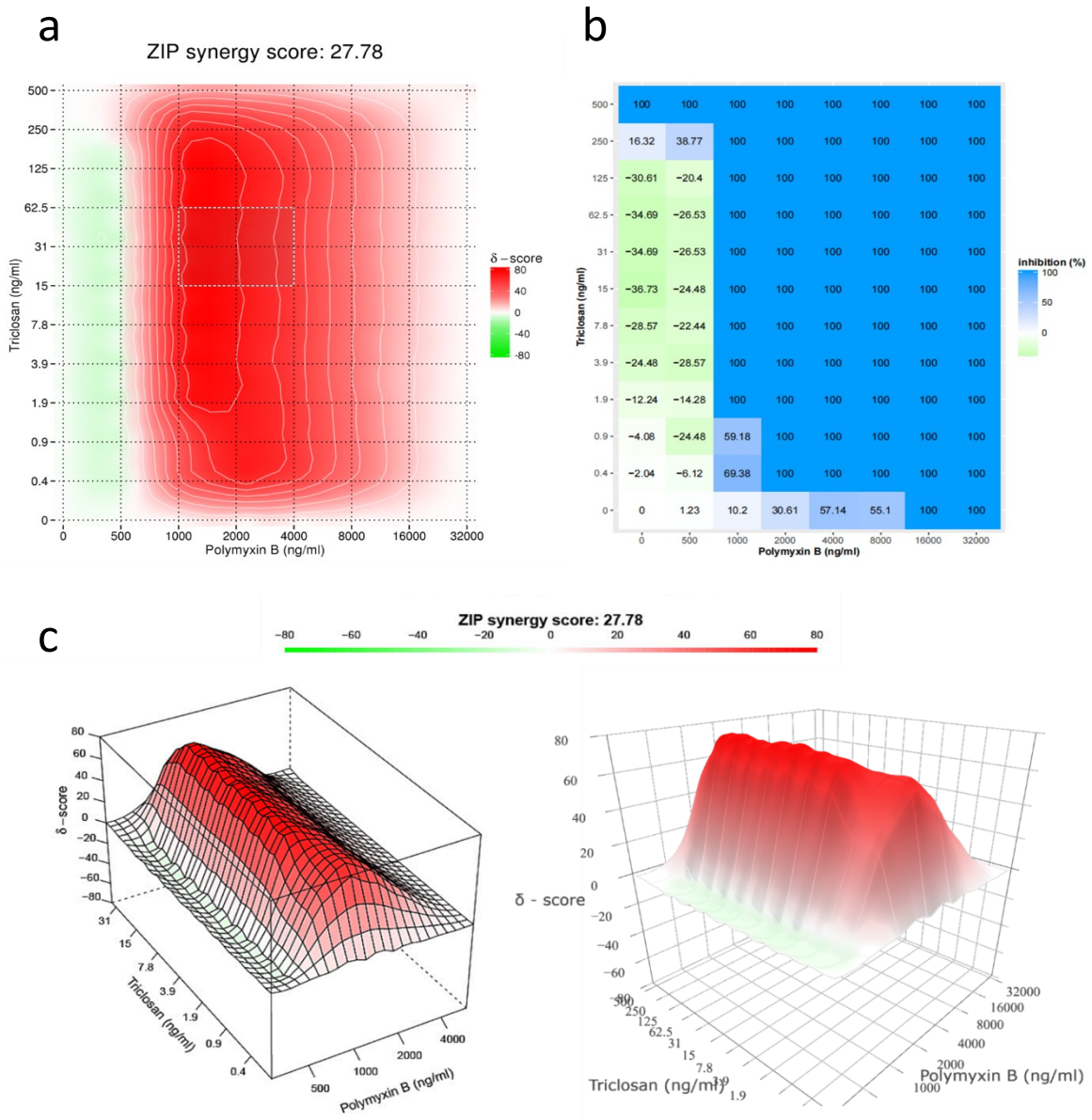

Fig 4: Synergistic interaction between triclosan and polymyxin B against *K. pneumoniae* was analyzed using checkerboard broth microdilution and visualized through synergy mapping. Regions of optimal synergy are highlighted across concentration ranges. (a). 2D synergy map displaying the δ-scores of triclosan and polymyxin B combinations. Red regions indicate strong synergy, with a ZIP synergy score of 27.78, confirming enhanced antimicrobial activity and the map highlights regions where optimal synergy is achieved across various concentrations, (b.) Heatmap showing inhibition percentages across various concentration combinations. Maximum inhibition (100%) is observed in specific drug pair concentrations, (c.) 3D visualization of the δ-scores, providing a topographical view of synergy intensity, with clear peaks representing optimal synergistic combinations.

2.4. Time-dependent eradication of colistin resistant *Klebsiella pneumoniae*

The time-kill assay results for colistin resistant *K. pneumoniae* demonstrated that neither triclosan (TCS) nor polymyxin B (PMB) at sub-MIC concentrations (1/8 MIC) exhibited significant antibacterial activity when used alone (Fig 5). Triclosan treatment (0.0625 µg/mL) allowed bacterial growth to progress similarly to the growth control, with counts reaching approximately 10^{11} CFU/mL by 24 hours. Similarly, polymyxin B treatment (2 µg/mL) showed minimal effect, with bacterial counts remaining close to those of the growth control throughout the experiment. In contrast, the combination of triclosan and polymyxin B at 1/8 MIC each displayed a potent synergistic effect. A significant reduction in bacterial counts was observed by 8 hours, with levels decreasing to approximately 10^3 CFU/mL by 24 hours. Notably, after 48 hours of combination treatment, no bacterial colonies were observed, indicating complete eradication of the bacterial population. These results highlight the potential of combining triclosan and polymyxin B at sub-MIC levels as a highly effective strategy against colistin resistant *K. pneumoniae*, achieving complete bacterial clearance where monotherapies failed.

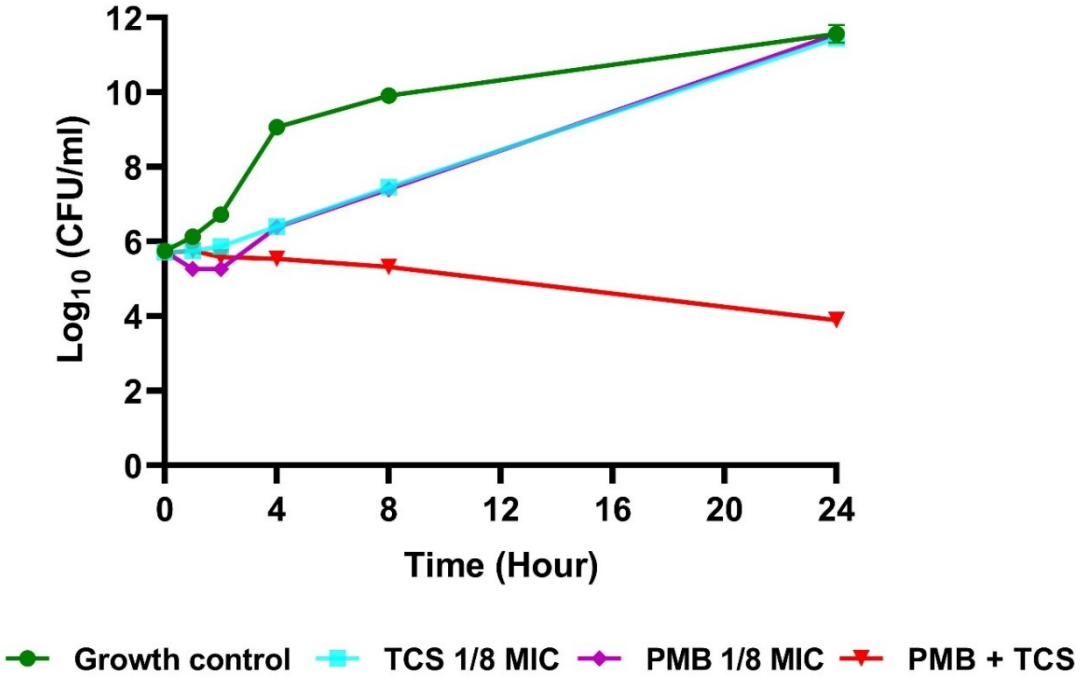

Fig 5: The time-kill assay shows the antimicrobial effects of triclosan, polymyxin B, and their combination at sub-MIC concentrations (1/8 MIC each) against colistin resistant *K. pneumoniae*. The x-axis represents time (0−24 hours), and the y-axis indicates bacterial counts

(Log_{10} CFU/mL). The growth control (green line) and triclosan alone (cyan line) both exhibit exponential bacterial growth, reaching approximately 10^{11} CFU/mL by 24 hours, while polymyxin B alone (magenta line) has minimal inhibitory effect, with bacterial counts close to the control. In contrast, the combination of triclosan and polymyxin B (red line) shows a strong synergistic effect, significantly reducing bacterial counts by 8 hours and achieving approximately 10^3 CFU/mL by 24 hours. The absence of colonies after 48 hours of combination treatment, although not shown in the graph, confirms complete bacterial eradication.

2.5. Combination prevents biofilm formation

Biofilms are a major challenge in treating infections caused by *K. pneumoniae*, as they provide a protective environment that enhances bacterial resistance. In this study, we evaluated the effects of sub-minimal inhibitory concentrations (sub-MICs) of polymyxin B (PMB) (2 µg/mL, 1/8 MIC) and triclosan (TCS) (0.0625 µg/mL, 1/8 MIC), individually and in combination, on biofilm formation. The crystal violet staining data, measured as absorbance at 595 nm, demonstrated that the PMB + TCS combination significantly inhibited biofilm formation compared to the untreated growth control (GC) (Fig 6). Individual treatments with PMB or TCS produced only minor reductions in biofilm formation, as their absorbance values were slightly lower than the GC but not markedly different. Visual inspection of the stained 96-well plate supported these findings, with wells treated with PMB or TCS alone showing staining levels similar to the GC, indicating minimal biofilm disruption. In contrast, the PMB + TCS combination-treated wells exhibited almost no staining, reflecting an almost complete inhibition of biofilm formation. These findings underscore the potent synergistic effect of combining PMB and TCS at sub-MIC levels, offering a promising strategy for combating biofilm-associated infections caused by *K. pneumoniae*.

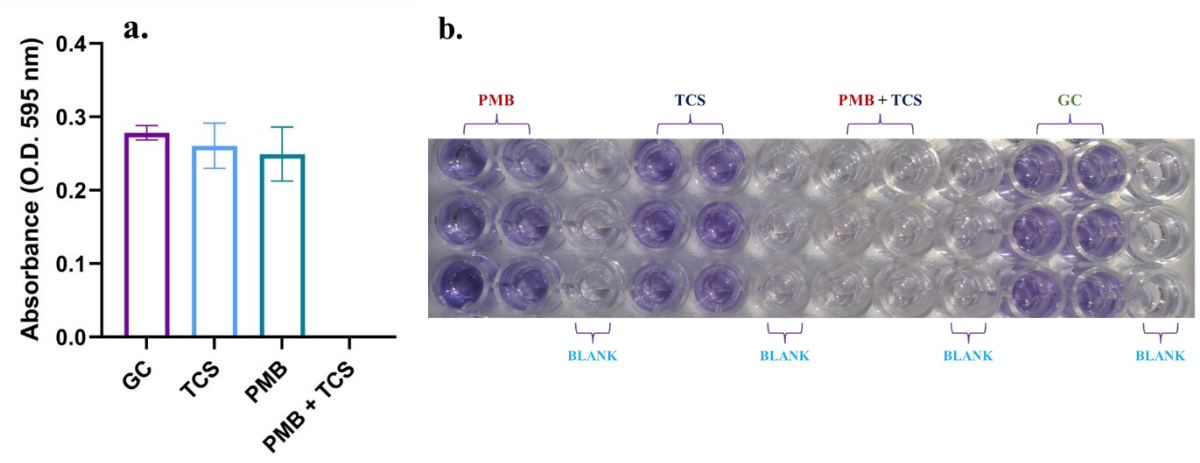

Fig 6: Crystal violet staining was used to quantify biofilm formation by *K. pneumoniae* in the presence of sub-minimal inhibitory concentrations (1/8 MIC) of polymyxin B (PMB, 2 µg/mL) and triclosan (TCS, 0.0625 µg/mL), individually and in combination, (a.) Absorbance data at 595 nm demonstrate that the PMB + TCS combination completely inhibited biofilm formation, while individual treatments with PMB or TCS resulted in biofilm levels only slightly lower than the growth control (GC). Data are shown as mean ± standard deviation, (b.) Visual confirmation of biofilm biomass via crystal violet staining in a 96-well plate. Wells treated with PMB + TCS show no visible staining, indicating no biofilm formation, while those treated with PMB or TCS alone exhibit staining similar to the GC. Blank wells serve as negative controls.

2.6. *Klebsiella pneumoniae* shows insignificant resistance evolution against combination therapy

The mutation frequencies in *K. pneumoniae* were analysed under selective pressure from polymyxin B, triclosan, and their combination at varying concentrations. Polymyxin B exhibited mutation frequencies of 4.6×10^{-9} and 1.07×10^{-9} at 4× MIC and 8× MIC, respectively, indicating a significant reduction in resistant mutants at higher drug concentrations. For triclosan, no resistant colonies were observed at 4× and 8× MIC range, with mutation frequency below the detectable threshold relative to the inoculum size. Similarly, the combination treatment did not result in detectable colonies at MIC level, signifying an absence of mutations under these conditions. It can be presumed that our combination therapy is promising, as there is no single-step mutation, indicating a very rare chance of resistance forming against the combination for *K. pneumoniae*. Furthermore, since no growth was

observed at 4× MIC of triclosan, it suggests that resistance against triclosan is also unlikely to develop.

Table 2: Single step mutation frequencies of *Klebsiella pneumoniae* under polymyxin B, triclosan, and combination

Drug	MIC (µg/ml)	Drug Conc. (µg/ml)	Inoculum (CFU/ml)	Single-Step Mutation frequency
Polymyxin B	16	64	2.8×10^{9}	4.6×10^{-9}
		128		1.07×10^{-9}
Triclosan	0.5	2	2.6×10^{9}	$< 2.6 \times 10^{-9}$
		4		$< 2.6 \times 10^{-9}$
PMB + TCS	—	16 + 0.5	2.7×10^{9}	$< 2.7 \times 10^{-9}$

2.7. Combination potentiates membrane disruption

The flow cytometry analysis conducted over a 4-hour treatment period reveals the impact of sub-MIC concentrations of triclosan and polymyxin B on the colistin resistant *K. pneumoniae* strain. Although the MIC for polymyxin B for this strain was 16 µg/mL, we used 1/8th of the MIC for both agents, triclosan (0.0625 µg/mL) and polymyxin B (2 µg/mL) to evaluate whether their combination could reverse this resistance. According to EUCAST guidelines, concentrations above 2 µg/mL for polymyxin B drug indicate resistance in *K. pneumoniae*, making this experiment particularly relevant for examining synergistic effects. PI staining (red dots), marking dead cells, shows notable differences across treatments (Fig 7). The untreated control sample exhibits low cell death, with only 11.2% PI positivity. Treatment with polymyxin B alone results in a modest increase in cell death to 27%, while triclosan alone leads to a slight decrease, with 7.4% PI positivity, suggesting limited bactericidal efficacy at this sub-MIC level. However, the combination of colistin and triclosan dramatically increases cell death to 58.3%, indicating a pronounced synergistic effect. The bar graph (Fig 6) showing PI-positive cell percentages underscores the substantial increase in cell death with the combination treatment, supporting the potential of triclosan and colistin together to sensitize colistin or polymyxin B resistance in this strain. These findings suggest that sub-MIC combinations of these agents could effectively enhance bactericidal activity and potentially overcome AMR in colistin resistant *K. pneumoniae*.

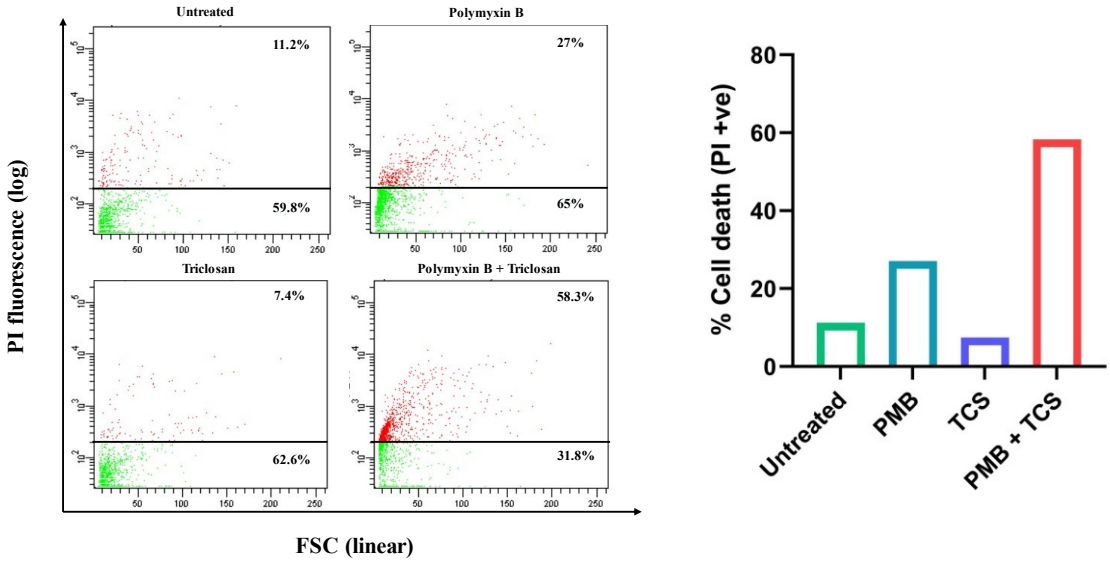

Fig 7: Flow cytometry analysis showing the effects of triclosan and polymyxin B, individually and in combination, on cell death in a colistin resistant *K. pneumoniae* strain. The four scatter plots represent untreated cells, cells treated with polymyxin B (2 µg/mL), cells treated with triclosan (0.0625 µg/mL), and cells treated with a combination of polymyxin B and triclosan at the same concentrations. Red dots indicate PI-positive (dead) cells, while green dots indicate live cells. Percentages in each plot represent the proportion of PI-positive cells, with the untreated control showing 11.2% cell death, polymyxin B alone at 27%, triclosan alone at 7.4%, and the combination treatment showing a significantly higher cell death rate of 58.3%, indicating a reversal of colistin resistance. The accompanying bar graph quantifies these percentages, demonstrating the enhanced bactericidal effect of the combination therapy compared to individual treatments.

2.8. Combination does not show cytotoxicity

The MTT assay results, as shown in the bar graph (Fig 8), demonstrate that treatment with polymyxin B, triclosan, and their combination (PMB + Triclosan) did not significantly impact cell viability in RAW 264.7 cells. Across all treatment conditions, including the control, cell viability remained above 90%, indicating minimal cytotoxicity. Specifically, the treatments with polymyxin B and triclosan, individually and in combination, did not differ significantly

from the control group, as confirmed by two-way ANOVA statistical analysis. These findings suggest that polymyxin B and triclosan are non-toxic to RAW 264.7 cells under the tested conditions, supporting their compatibility for potential therapeutic applications without compromising cellular integrity.

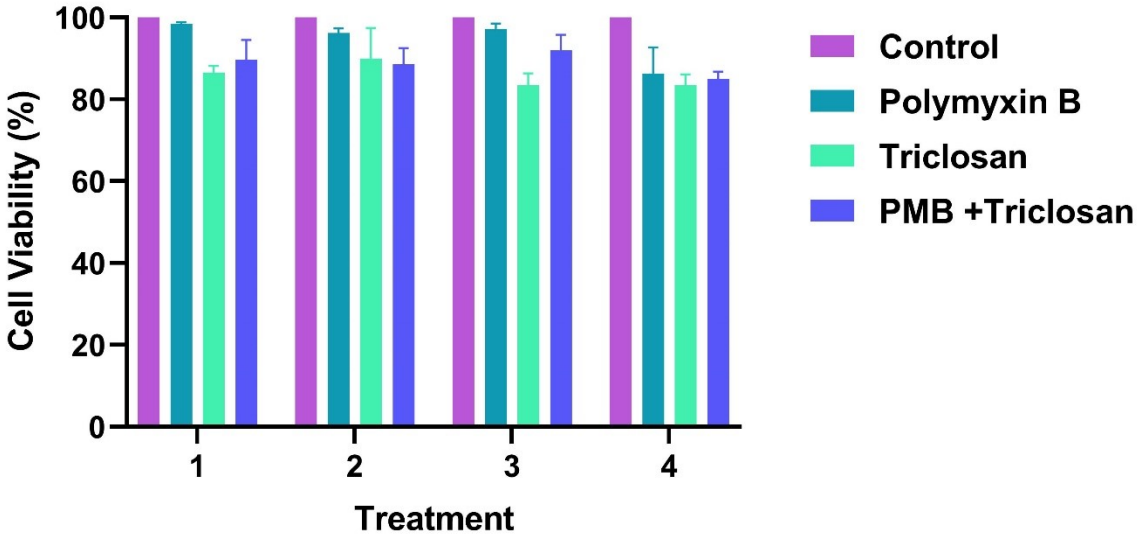

Fig 8: Cell viability of RAW 264.7 cells following treatment with varying concentrations of triclosan, polymyxin B, and their combination. Cell viability was assessed using an MTT assay. In Group 1, cells were treated with triclosan at 0.031 µg, polymyxin B at 2 µg, and a combination of 2 µg polymyxin B and 0.031 µg triclosan. In Group 2, concentrations were increased to 0.125 µg triclosan, 4 µg polymyxin B, and a combination of 4 µg polymyxin B and 0.125 µg triclosan. In Group 3, cells received 0.5 µg triclosan, 8 µg polymyxin B, and a combination of 8 µg polymyxin B with 0.5 µg triclosan. In Group 4, the highest concentrations were tested: 1 µg triclosan, 16 µg polymyxin B, and a combination of 16 µg polymyxin B and 1 µg triclosan. Error bars represent standard deviation.

2.9. *K. pneumoniae* isolates do not have a significant mutation in *fabI* gene

Since there are several reports hypothesize a resistance against triclosan as it released in the environment after its applications. Thus, we attempted to examine the potential mutation in the FabI encoding gene because FabI is an exclusive target for triclosan binding. There are three potential mutations reported in the active site of FabI that may reduce the affinity of triclosan and subsequently be resistant to triclosan. Among these reported residues, Gly93 has direct

interaction through hydrogen bonds while Met159 and Phe203 interact through hydrophobic and aromatic ring stacking attractions. Therefore, we focused on identifying mutations in these residues in *K. pneumoniae* isolates. Surprisingly we did not find significant mutation of these three residues in isolates.

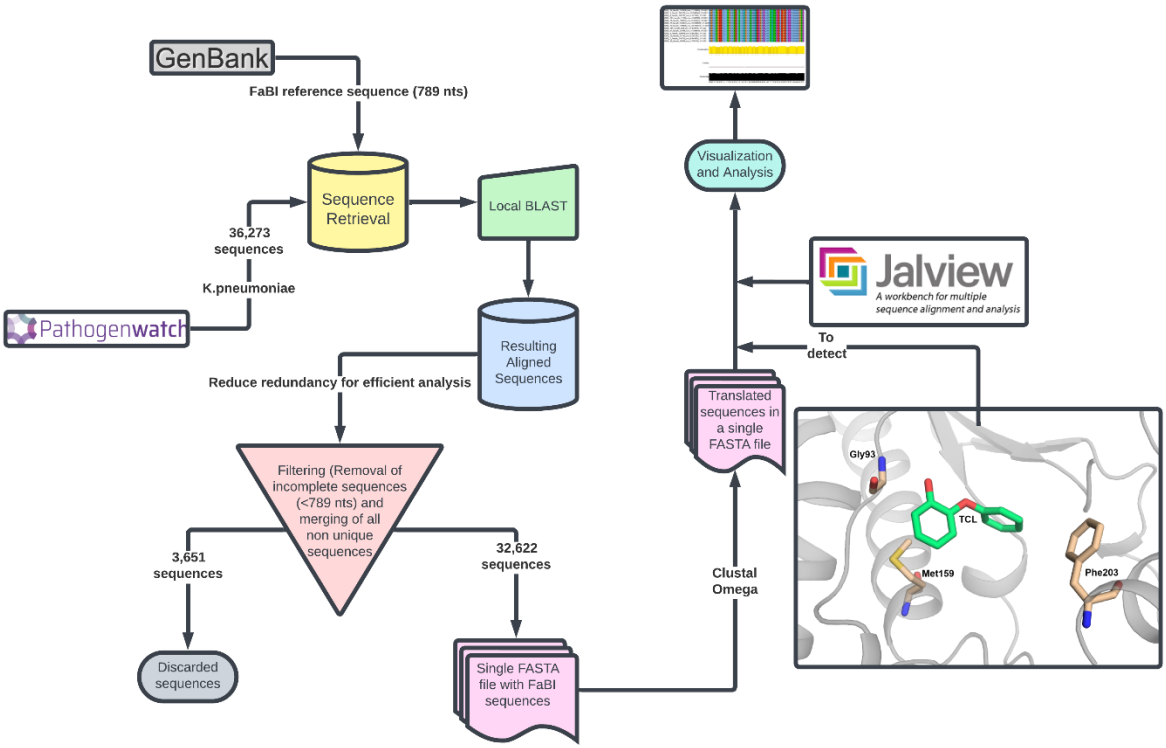

Figure-9: A schematic workflow for detection of mutations in triclosan binding site residues of FabI, a target for triclosan binding. Sequences of FabI encoding gene were aligned and examined for mutations in the three binding site residues of FabI.

3. Discussion

Gram-negative bacteria with multi-drug resistance (MDR) pose a significant global threat (Breijyeh et al., 2020), with *Klebsiella pneumoniae* emerging as a leading cause of nosocomial and healthcare-associated infections among other pathogens (Alcántar-Curiel et al., 2018). In the context of AMR, *K. pneumoniae* strains producing extended-spectrum beta-lactamases (ESBLs) and showing carbapenem resistance have emerged as a significant concern, exhibiting resistance to the majority of clinically effective antibiotics (Karampatakis et al., 2023; Romyasamit et al., 2024). To address this crisis, polymyxins and colistin have been extensively utilized as last-resort therapies for critically ill patients, particularly in ICU patients (Morris

and Cerceo, 2020; Petrosillo et al., 2019). Alarmingly, resistance to colistin has also emerged, further exacerbating the threat and underscoring the urgent need for vigilant monitoring of its prevalence and underlying mechanisms (Goel et al., 2014).

Our study focused on the global prevalence of colistin resistance in *K. pneumoniae* from 2009 to 2023, revealing a rapid escalation in resistance rates during this period. By 2022, colistin-resistant *K. pneumoniae* accounted for over 13% of cases involving *Klebsiella*-mediated infections. This alarming trend is exacerbated by the rise of plasmid-mediated mobile colistin resistance (*mcr*) genes, which facilitate the horizontal transfer of resistance across bacterial strains and species. The first case of *mcr-1*-mediated colistin resistance in *K. pneumoniae* was reported in 2016 (Liu et al., 2016), and our findings corroborate this timeline, showing a sharp increase in *mcr-1* prevalence in subsequent years, peaking in 2023. Despite the significance of *mcr-1*, our data indicate that the majority of colistin-resistant *K. pneumoniae* strains acquire resistance through alternative mechanisms. The literature identifies mutations in the *mgrB* gene, which encodes a transmembrane regulatory protein, as well as mutations in genes linked to the two-component regulatory systems PmrA/PmrB and PhoP/PhoQ, as major contributors to colistin resistance (Poirel et al., 2017a; Wright et al., 2015). Enhanced expression of *pmrA, pmrB, pmrD, pmrK, phoP,* and *phoQ* genes has also been consistently observed in colistin-resistant isolates (Borsa et al., 2019). Given the gravity of the situation, further genomic investigations are crucial to uncover novel markers and traits associated with colistin resistance. The continent-wise analysis of colistin resistance in *K. pneumoniae* underscores the uneven distribution of resistance across the globe. The high burden in Europe and North America suggests a need for enhanced AMR control measures in these regions while emerging resistance in Asia highlights the risk posed by widespread antibiotic use in densely populated areas. The lower reported cases in Africa and South America emphasize the need for improved surveillance to better understand the true extent of AMR in these regions. These findings stress the importance of global collaboration in AMR monitoring and the implementation of region-specific strategies to mitigate the spread of colistin resistance.

In the gravity of the situation, combination therapy has emerged as a swift strategy to combat MDR gram-negative pathogens(Tängdén, 2014). Clinically validated combinations, such as beta-lactam antibiotics paired with aminoglycosides, have proven effective in treating severe infections caused by gram-negative bacteria (Tamma et al., 2012). In the face of rising resistance to even last-resort drugs, combination therapies involving polymyxin B have shown great potential to enhance and preserve antimicrobial efficacy. For instance, the combination

of polymyxin B and trimethoprim is already clinically approved for treating eye infections, including acute bacterial conjunctivitis and blepharoconjunctivitis (Wagner, 1995). Researchers are actively exploring additional combinations, such as polymyxin B with netropsin, which has demonstrated activity against MDR *Acinetobacter baumannii* (Chung et al., 2016). Similarly, the combination of polymyxin B and minocycline has shown promise against carbapenem-resistant *K. pneumoniae* (Zhao et al., 2020). Other synergistic combinations, including polymyxin B with tigecycline and polymyxin B, paired with avibactam-based beta-lactam/beta-lactamase inhibitors (BL-BLIs), have also displayed significant potential against gram-negative bacteria (Li et al., 2022).

In our study, we investigated the synergistic effects of polymyxin B combined with the established antibacterial agent triclosan. Remarkably, this combination significantly reduced the minimum inhibitory concentration (MIC) of polymyxin B to 1 μg/mL, with a fractional inhibitory concentration index (FICI) of 0.0663—an improvement compared to other combinations. Given that both agents are proven antibacterial compounds, this combination holds considerable potential for clinical application pending further trials and research.

The time-kill assay (TKA) plays a crucial role in preclinical modelling, providing detailed insights into the interaction between antimicrobial agents and bacteria (Bax et al., 2017). It offers valuable data on the rate of bacterial eradication over time and identifies the optimal concentration ranges for rapid or sustained activity (Filimonova et al., 2021). To evaluate the preclinical potential of the triclosan and polymyxin B combination, we conducted TKA, which showed promising results. According to EUCAST guidelines, the resistance cutoff value for polymyxin B is ≥2 μg/mL (*EUCAST. European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameters, version 7.1*, 2017). In this study, we utilized polymyxin B at 1/8 of its MIC (2 μg/mL) to test the combination effect within the susceptible range. Triclosan was used at a similarly low concentration of 1/8 MIC (0.0625 μg/mL). The results were highly encouraging: within 24 hours, the bacterial load was reduced by approximately 2 log10, and by 48 hours, complete bacterial eradication was observed. This highlights the effectiveness of our combination even at low, clinically relevant concentrations of polymyxin B, supporting its potential as a viable therapeutic candidate.

One major challenge in treating *K. pneumoniae* infections is its ability to form biofilms (Guerra et al., 2022). Biofilms create a protective matrix on surfaces such as tissues, wounds, or medical devices, significantly enhancing bacterial resistance to antibiotics and the immune system

(Shree et al., 2023). Remarkably, our combination therapy demonstrated exceptional efficacy in the biofilm adherence assay at the same susceptible range of polymyxin B used in TKA. While individual agents showed minimal inhibition of biofilm growth, comparable to the untreated control, the combination completely inhibited biofilm formation within 24 hours. This finding adds further support to the combination of triclosan and polymyxin B for further consideration.

Another important factor in developing new antimicrobial agents is assessing their evolutionary potential to cause resistance in target organisms (Martinez and Baquero, 2000; Uddin et al., 2021). The frequency of resistance (FoR) assay helps determine the chance of resistant strains emerging in clinical settings (Evans and Titlow, 1998). In this study, polymyxin B alone at 8×MIC exhibited single-step mutation frequency. However, no mutations were observed when using the combination of polymyxin B (16 µg/mL) and triclosan (2 µg/mL) in MIC range, indicating a significantly reduced risk of resistance development.

Non-significant cytotoxicity observed in RAW 264.7 cells represent another strong point in favour of the combination, suggesting that it maintains cellular integrity during treatment. Additionally, enhanced bacterial cell death observed in the flow cytometry-based cell viability assay further corroborates our findings, highlighting the efficacy of the combination in promoting bacterial eradication while maintaining minimal toxicity to cells.

Triclosan is widely used in several personal products as an antibacterial agent, thus there are various *in vitro* studies have reported describing that it can cause resistance by mutating binding site residues in its target FabI enzyme (Heath et al., 1999; Sivaraman et al., 2003). Therefore, we examined publicly available genomes of *K. pneumoniae* isolates to detect these mutations. Remarkably, we could not find any significant mutation for potential binding site residues in the *K. pneumoniae* FabI enzyme. It suggests that triclosan may not cause mutations in FabI conferring for triclosan resistance in *K. pneumoniae.* Furthermore, our frequency of resistance assay for triclosan also indicates that triclosan resistance was not developed by applying a subinhibitory concentration of triclosan. These findings show that triclosan in combination with polymyxin B may not cause mutation in FabI for resistant development.

Collectively, these findings underscore the promising potential of the triclosan and polymyxin B combination as a novel therapeutic approach, suggesting the need for further exploration in the antibacterial drug development pipeline.

4. Conclusions

The rise of AMR among Gram-negative pathogens such as *Klebsiella pneumoniae* poses a serious global health challenge, particularly with increasing resistance to “last-resort" antibiotics like colistin and polymyxins. Our study provides an overview of current trends in colistin resistance in *K. pneumoniae* with respect to prevalence, mechanisms, and geographical distribution. Global prevalence data revealed a sharp increase in colistin resistance rates from 2009 to 2023, with *mcr-1* mediated resistance cases being relatively low compared to non-*mcr-1* mechanisms, indicating the importance of exploring novel chromosomal or genomic resistance markers beyond those currently reported. Regional variations in resistance, with high rates in Europe and North America and emerging trends in Asia, emphasize the need for region specific AMR controlling strategies including antibiotic stewardship programs. Furthermore, we investigated a therapeutically promising combination therapy comprising polymyxin B and triclosan. This combination significantly reduces the MIC of polymyxin B in colistin resistant *K. pneumoniae* while revealing strong synergistic activity, as confirmed by time-kill assays which showed complete bacterial eradication within 48 hours at clinically susceptible concentrations of polymyxin B. The combination also revealed exceptional efficacy by completely inhibiting their biofilm formation within 24 hours, which is crucial due to the role of biofilms in antibiotic resistance. Additionally, cytotoxicity testing on RAW 264.7 cells revealed non-significant toxicity, supporting its safety profile, while flow cytometry studies confirmed enhanced bacterial cell death with the combination. Genomic analysis of *K. pneumoniae* revealed no significant mutations in the FabI binding site which could trigger triclosan resistance, further supporting the feasibility of this combination. While these findings are promising, this combination was only tested in *Klebsiella*, and testing in other Gram-negative bacteria is also required for broader-range validation, ensuring its applicability across diverse MDR pathogens.

5. Materials and methods

5.1. Global colistin resistance analysis

A total of 40,445 *Klebsiella pneumoniae* whole-genome sequences were downloaded from the PathogenWatch database (Argimón et al., 2021), spanning the years 2009 to 2023. From this dataset, sequences were systematically filtered to identify isolates annotated with colistin resistance. The filtering process focused on sequences marked as colistin-resistant, identified

by examining the antibiotic resistance profiles for each sequence within the PathogenWatch platform. From these resistant sequences, we screened for the *mcr-1* gene, which is known to contribute to colistin resistance (Granata and Petrosillo, 2017). To confirm the presence of the *mcr-1* gene, we utilized the Basic Local Alignment Search Tool (BLAST) with optimized parameters to enhance detection sensitivity (Altschul et al., 1990). Specifically, we set a word size of 11 to improve the tool's capacity to identify short or subtle sequence alignments, which are characteristic of resistance genes like *mcr-1*. We applied an E-value cutoff of 1e-5 to exclude lower-confidence alignments, thereby retaining only highly reliable matches. Additionally, we imposed a minimum percent identity threshold of 90%, focusing our analysis on sequences with high similarity to known *mcr-1* variants. To manage the impact of insertions or deletions-common within mobile genetic elements-we implemented gap penalties of -2 for gap opening and -1 for gap extension. The BLOSUM62 scoring matrix (Henikoff and Henikoff, 1992) was employed to optimize the detection of conserved regions within the *mcr-1* gene. Furthermore, low-complexity regions were excluded from the analysis to prevent misleading alignments. Only alignments covering 70–80% or more of the query length were deemed significant, ensuring a high-confidence dataset and robust identification of *mcr-1* sequences.

Following the identification of *mcr-1* positive sequences, all data were organized on a yearly basis from 2009 to 2023. This included individual cases reported each year, as well as the total number of colistin-resistant strains identified annually. Using these data, the percentage of colistin-resistant infections for each year was calculated. Additionally, we identified the unique cases with only *mcr-1* positive sequences and determined the proportion of colistin resistance attributable specifically to the presence of the *mcr-1* gene in *K. pneumoniae* isolates. All data were visualized and analyzed using GraphPad Prism (version 9.5.1) to facilitate graphical representation and comparison between the number of colistin-resistant cases and the prevalence of *mcr-1* in the positive isolates across the study period.

5.2. Geographic distribution of colistin resistance

K. pneumoniae sequences exhibiting colistin resistance, previously retrieved from the Pathogenwatch database for the period 2009 to 2023, were analysed to determine the geographic distribution of resistance. Along with the sequences, all available metadata were downloaded, providing detailed geographic information regarding the origin of each sequence.

This allowed for classification by continent, including North America, Europe, Asia, Africa, South America, and Australia.

To obtain continent-based data, colistin-resistant sequences were aggregated and grouped according to their continent of origin. Although some metadata was incomplete, the analysis was conducted based on the available data. The data were then summarized with case counts sorted by continent to generate cumulative totals for the period 2009 to 2023. These results were visualized on a world map, with each continent highlighted according to the number of colistin-resistant cases reported.

5.3. Strains and culture

Confirmed *K. pneumoniae* strains were obtained from clinical isolates collected in a hospital setting, where initial confirmatory testing was performed. Strain identity was verified through biochemical tests, including lactose fermentation, urease production, citrate utilization, and indole production. Further confirmation of multidrug resistance (MDR) profiles, specifically for resistance to colistin and polymyxin B, was achieved using the VITEK automated system (BioMérieux, France). The hospital generated detailed VITEK reports that outlined the resistance profiles for each isolate, providing accurate identification of MDR.

Additionally, PCR was conducted with *mcr-1* gene-specific primers to confirm colistin resistance at the genetic level. As per EUCAST guidelines, broth microdilution assays were performed, as detailed below, to obtain final confirmation of colistin resistance and to corroborate PCR results.

Inoculum optimization for *K. pneumoniae* was performed using the laboratory strain *K. pneumoniae subsp. pneumoniae* (MTCC109) as a standard. Primary cultures of this strain were prepared in triplicate and incubated overnight with shaking at 150 rpm at 37°C in Mueller-Hinton Broth (MHB). After incubation, cultures were diluted with fresh MHB to achieve a range of optical densities (OD) at 600 nm, spanning from 0.1 to 1.0. Samples from each culture, adjusted to these specific OD values, were subjected to serial dilutions (10^{-4} to 10^{-10}), with higher OD cultures undergoing greater dilution to ensure countable colony-forming units (CFUs) on agar plates.

Using the drop plate method, 5 µL aliquots from each dilution were plated onto MHB agar in triplicate and incubated overnight to allow colony formation. CFUs were enumerated the

following day, and the data were plotted to establish a correlation between CFU counts and their respective OD values. This approach provides accurate CFU estimation based on OD, supporting optimal inoculum preparation for experimental protocols involving *K. pneumoniae*.

5.4. Determination of minimum inhibitory concentration (MIC)

Resistance to polymyxin B and the MIC was assessed using the Broth Microdilution (BMD) method. This method, recommended by ISO 20776-1 ("ISO 20776-1:2019 - Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices — Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases," n.d.) and endorsed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), is the standard approach for determining MIC values of polymyxins (Poirel et al., 2017b).

The process followed the standardized protocol outlined by EUCAST. A working stock solution of polymyxin B was prepared at a 2-fold higher concentration (64 µg/ml) in freshly autoclaved MHB. The assay was conducted in a 96-well microdilution plate, with 100 µl of MHB added to each well of a row, except for the first. In the first well, 200 µl of the highest polymyxin B concentration (64 µg/ml) was added. Serial 2-fold dilutions were then carried out by transferring 100 µl from the first well to the next, continuing until 2-fold of the desired concentration range of 0.5 µg/ml to 32 µg/ml was achieved. The last 100 µl from the final well was discarded, and a control well without the antibiotic was included to check for normal bacterial growth. Overnight cultures of *K. pneumoniae*, adjusted to an optical density (OD) of 0.1 (corresponding to ~10^8 CFU/ml and previously optimized through bacterial growth estimation methods), were further diluted 100-fold to reach a bacterial concentration of 10^6 CFU/ml. A 100 µl inoculum of this bacterial suspension was added to each well, resulting in a final volume of 200 µl per well. The 2-fold concentration of the drugs in MHB was appropriately diluted to achieve the desired experimental concentrations, while also maintaining the inoculation density at 5×10^5 CFU/ml, a standard inoculation amount for performing MIC experiments.

5.5. Checkerboard assay

The interaction between the combination drug polymyxin B and triclosan was assessed using the checkerboard assay method in a 96-well plate, with modifications tailored to this combination. Initially, a polymyxin B antibiotic working stock was prepared at concentrations 2-fold and 4-fold higher than the desired concentration in MHB. For instance, if the desired concentration of polymyxin B in row A was 32 µg, working stocks of 64 µg and 128 µg were prepared. Freshly autoclaved MHB was dispensed at 100 µl into each well, excluding row A. Subsequently, 200 µl of the 2-fold working stock of polymyxin B was added to wells 1 to 11 in row A, with the 4-fold working stock added to the 12th well. This arrangement ensured that the subsequent addition of another drug in the 12th column would result in a dilution from 4-fold to 2-fold, maintaining symmetry along the row for the concentration of polymyxin B. Serial dilutions were then carried out from rows B to G, with an excess 100 µl discarded from row G to maintain a final volume of 100 µl in each well (excluding row H), thus achieving a wide concentration range of polymyxin B from 0.5 µg to 32 µg in the final 96-well plate following inoculation (as shown in Fig. 1).

In the subsequent step, the second drug, triclosan, was prepared as a main stock solution DMSO, with a subsequent sub-stock or working stock prepared in MHB at a 4-fold higher concentration than the highest desired range of triclosan. For instance, if the highest desired range was 0.5 µg, a 2 µg working stock of triclosan was prepared. Then, 100 µl of the working stock of triclosan was added to the 12th column, already containing 100 µl of serially diluted polymyxin B, resulting in a total volume of 200 µl and halving the concentrations of triclosan and polymyxin B from 4-fold to 2-fold higher. Serial dilutions of triclosan were performed from the 12th column to the 2nd column, with excess 100 µl of media discarded from the 2nd column to ensure a uniform volume of 100 µl throughout the plate, thus creating a wide concentration range of triclosan from 0.0004 µg to 0.5 µg in the final plate. This process facilitated the determination of the MIC for each individual drug as well as for the combination. For instance, the 1st column contained only polymyxin B, while row H contained only triclosan, and the well in row H-1 served as a growth control with no drug present (Shown in Fig).

An overnight growth culture of *K. pneumoniae*, known to be polymyxin B and colistin-resistant, was utilized for inoculation. The culture was diluted according to the pre-described method to achieve a final inoculum of 10×10^5 CFU/ml. Subsequently, 100 µl of the inoculum was added to each well to achieve a final volume of 200 µl and a final bacterial inoculum of 5×10^5 CFU/ml. Additionally, it is important to note that with the addition of the inoculum, the drug concentrations in each well were halved, aligning with the desired concentration range for

the assay. This step ensured the maintenance of consistent experimental conditions and facilitated the evaluation of antimicrobial efficacy across the intended concentration spectrum. The 96-well plate was subsequently incubated for 20-24 hours at 37 °C to observe the final outcomes. After the incubation period, absorbance values were collected at 600 nm and plotted as a heatmap to facilitate better understanding and calculation of the fractional inhibitory concentration (FIC) using the formula:

$$FIC = \left(\frac{MIC_{Combination}}{MIC_{Triclosan}}\right)_{Triclosan} + \left(\frac{MIC_{Combination}}{MIC_{Polymyxin\ B}}\right)_{Polymyxin\ B}$$

An FIC index of 0.5 or less indicates synergistic action between the antimicrobial agents, where their combined effect exceeds the sum of their individual effects. FIC indices falling between 0.5 and 4 suggest additivity, indicating that the combined effect is roughly equivalent to the sum of their individual effects. Conversely, an FIC index exceeding 4 indicates antagonism, signifying interference between the antimicrobial agents leading to a diminished combined effect compared to their individual effects.

5.6. Synergy data analysis

Absorbance data from the checkerboard assay were analysed using the Synergy Finder web application (Version 3.0) (Ianevski et al., 2022). Data were organized according to the reference model specified in the application, and due to software limitations, drug concentrations were converted from µg/ml to ng/ml. Drug combination responses were expressed as percent inhibition, calculated using the formula:

$$\%\ \text{Inhibition} = \frac{(\text{Control OD} - \text{Sample OD})}{(\text{Control OD})} \times 100$$

A four-parameter logistic regression (LL4) model was applied for curve fitting, and the Zero Interaction Potency (ZIP) model was used to assess synergy. Analysis results were then reported for interpretation.

5.7. Time-kill kinetics assay

The Time-kill assay was conducted to evaluate the viability of *K. pneumoniae 2110* under combination therapy with triclosan and polymyxin B. According to the Clinical and Laboratory

Standards Institute (CLSI), the susceptible breakpoint MIC for polymyxin B and colistin in Enterobacterales is ≤2 μg/ml (*Clinical and Laboratory Standards Institute (CLSI) Performance standards for antimicrobial susceptibility testing. 30th ed. Wayne, PA: CLSI; 2020. CLSI supplement M100*, n.d.). For this assay, the concentration of polymyxin B was set at 1/8 of the MIC, to get a final concentration of 2 μg/ml. Similarly, triclosan was also used at 1/8 of its MIC. Initial working stock solutions for both polymyxin B and triclosan were prepared at concentrations 200-fold higher than the desired assay concentrations, using freshly autoclaved MHB for polymyxin B and dimethyl sulfoxide (DMSO) for triclosan.

Freshly autoclaved tubes containing 2.5 ml of MHB were prepared for the assay. Four experimental conditions were set: one tube containing 1/8 MIC of polymyxin B, one containing 1/8 MIC of triclosan, one with a combination of both drugs and a control tube without any drug to allow for growth observation. To each tube, 25 μl of the respective working stock solution (polymyxin B or triclosan) was added to 2475 μl of MHB, achieving a 100-fold dilution for the individual treatments. For the combination therapy, 25 μl of each working stock (polymyxin B and triclosan, totalling 50 μl) was added to the MHB, maintaining the same 100-fold dilution.

Subsequently, the *K. pneumoniae* bacterial inoculum was adjusted to 10^6 CFU/ml using the previously described method, and an equivalent volume of 2.5 ml of the inoculum was added to each tube. This step ensured the attainment of the desired concentration of triclosan and a standardized bacterial inoculation count of 5×10^5 CFU/ml. All tubes were then incubated at 37°C with 150 rpm. At predetermined time intervals of 1, 2, 4, 8, and 24 hours, samples were collected from each tube. Depending on the time elapsed and the turbidity of the tube, samples were serially diluted up to 10^{-8} to facilitate readable CFU count on agar plates. Using the drop plate method, 5 μl aliquots from each serially diluted sample were plated onto MHB agar plates. For samples with higher concentrations of triclosan, aliquots were plated without dilution to accurately determine the rate of bacterial killing. All procedures, including triclosan dilution, bacterial inoculation, incubation, sampling, and CFU counting, were carried out independently in three sets of tubes or in triplicates to ensure experimental reproducibility and facilitate a comprehensive assessment of the efficacy of triclosan against *K. pneumoniae* over time and concentration.

All plates were incubated for 24 hours to enable proper CFU count determination. CFU counts for each concentration at each time point were calculated and converted into a logarithmic scale. The effectiveness of combination therapy over time and concentration was observed and

recorded by plotting the value of log CFU against time points. All data analysis and visualization were performed using GraphPad Prism.

5.8. Adherent biofilm formation assay

Biofilm formation was quantified by measuring the absorbance at 595 nm (A595) of crystal violet-stained adherent cells, following a previously described method with slight modifications (Mandell et al., 2019). *K. pneumoniae* was initially cultured in 10 mL of LB broth and incubated overnight at 37°C. The overnight culture was then diluted to the optimal OD for inoculation, as determined by prior growth estimation protocols. Sub-inhibitory MIC concentrations of polymyxin B (2 µg/ml, equivalent to 1/8 MIC), triclosan (0.0625 µg/ml, also 1/8 MIC), and a combination of both were prepared in LB broth, resulting in a total volume of 100 µL per well. A 100 µL aliquot of bacterial suspension at 10^6 CFU/ml was then added to each well, resulting in a 2-fold dilution and a final inoculum concentration of 5×10^5 CFU/ml. Growth control wells and blank controls were included by adding 100 µL LB with 100 µL of bacterial culture and 200 µL of LB alone, respectively. The plates were incubated at 37°C for 24 hours (without agitation). Following incubation, the culture media was carefully removed from each well, and the wells were washed three times with distilled water to eliminate non-adherent or planktonic cells. Biofilms were stained by adding 200 µL of 0.01% crystal violet solution to each well, allowing the adherent cells to be properly stained. After 15 minutes, the excess stain was removed by washing twice with sterile water. Crystal violet retained by adherent biofilm cells was then solubilized using 30% glacial acetic acid, and absorbance at 595 nm was measured to quantify biofilm formation.

5.9. Frequency of resistance analysis

The spontaneous single-step mutation rates for *K. pneumoniae* were determined by growing bacteria in antibiotic-free MHB broth overnight (concentration of approximately 10^9 CFU/ml) and plating 100 µl aliquots on Mueller-Hinton (MH) agar plate containing 4 x MIC, 8 x MIC of polymyxin B and triclosan individually, for the combination testing the MH agar plates were prepared in the MIC range (16µg PMB + 0.5µg TCS). A growth control was taken by serially diluting the overnight culture to 10^7 to obtain countable colonies on MH agar plates, all plates

were incubated for 24 to 48 hrs. All experiments were performed in triplicates. The resistance frequency was calculated using the following formula (Evans and Titlow, 1998):

$$\text{Resistance Frequency} = \frac{\text{(The number of colonies in the antibiotic plate)}}{\text{(Number of colonies in growth control plate)}}$$

5.10. Cell viability assay

Clinical strains of *K. pneumoniae* were initially cultured overnight in MHB at 37°C. The following day, the bacterial suspension was adjusted to an optimized optical density corresponding to a final concentration of approximately 10^8 CFU/mL, as previously determined. For the treatment phase, bacterial cells were exposed to sub-inhibitory concentrations of triclosan (0.0625 µg/mL, or 1/8 of the MIC) and polymyxin B (2 µg/mL, or 1/8 of the MIC). An additional combination treatment, maintaining the same concentrations of both agents, was prepared to assess potential synergistic effects. A control tube, containing the untreated bacterial culture, was included as a growth reference. All treatment and control tubes were incubated at 37°C for 4 hours.

Upon completion of the incubation period, 1 mL aliquots were withdrawn from each tube, including the control, and transferred into individual microcentrifuge tubes. To remove any residual antimicrobial agents, cells were pelleted by centrifugation at 7,000 *g* for 7 minutes, the supernatant was discarded, and the bacterial pellet was resuspended in 500 µL of sterile, syringe-filtered (0.22 µm) 1X phosphate-buffered saline (PBS). A single wash step was performed by centrifuging the resuspended cells again at 7,000 *g* for 7 minutes, discarding the supernatant, and resuspending the pellet in fresh 1X PBS. To assess cell viability, the washed bacterial suspensions were stained using SYTO 9 (Thermofisher Scientific, S34854) and propidium iodide (Thermofisher Scientific, P3566) to distinguish live and dead cells. Staining was conducted by incubating the cell suspensions in the dark for 30 minutes. Following incubation, samples were analyzed using flow cytometry (BD FACS Canto II) to quantify the proportion of dead cells across each treatment condition, using event counts to assess bacterial viability.

5.11. MTT cytotoxicity assay

RAW 264.7 cells were cultured by seeding in a 96-well plate at a density of 10^4 cells/well in 100 µl of complete cell culture medium, followed by incubation at 37 °C with 5% CO_2 for 24 hours to allow cell attachment and recovery. After incubation, the cells were treated with a range of triclosan and polymyxin B concentrations to assess dose-dependent effects, as well as combination treatments of both agents. Specifically, four concentrations of triclosan were used: 0.031 µg, 0.125 µg, 0.5 µg, and 1 µg per well, along with four concentrations of polymyxin B: 2 µg, 4 µg, 8 µg, and 16 µg per well. For the combination treatments, cells received simultaneous doses of both compounds at various levels, including the highest concentrations of each agent: 16 µg of polymyxin B combined with 1 µg of triclosan. Additional combination treatments included 2 µg of polymyxin B with 0.031 µg of triclosan, 4 µg of polymyxin B with 0.125 µg of triclosan, and 8 µg of polymyxin B with 0.5 µg of triclosan.

Following the 24-hour treatment period, the medium in each well was carefully aspirated and replaced with 100 µl of fresh medium, followed by the addition of 10 µl of a 12 mM MTT stock solution (Himedia, India) prepared in sterile PBS. Cells were then incubated at 37°C with 5% CO_2 for 3 hours to allow cellular reduction of MTT to formazan crystals. After this incubation, each well received 100 µl of a prepared solution of 0.01 M HCl in 10% SDS, which was added gently to dissolve the formazan crystals, facilitating measurement of the metabolic activity of the cells. The plates were then incubated for an additional 30 minutes to 1 hour at 37°C to ensure complete dissolution. Finally, each well was mixed by gentle pipetting, and the absorbance was measured at 570 nm to quantify the relative cell viability across the different treatment groups.

5.12. *fabI* gene variant analysis

Whole genome sequences of *K. pneumoniae* were obtained from PathogenWatch, a public genomic sequence repository and database. A total of 36,273 samples were taken (16th December 2023). The reference DNA sequence of *fabI* gene was obtained from *Klebsiella pneumoniae* subsp. pneumoniae MGH 78578 (GenBank Accession number CP000647.1:1441037-1441825). Local BLAST was carried out using the reference *fabI* gene sequence as a query, against the 36,273 samples to obtain the *fabI* sequence of each sample. Sequences with missing nucleotide residues and multiple gaps were omitted with a focus on the complete sequence length. After the removal, we were left with 32,622 sequences. The remaining sequences were then extracted in a single FASTA format file followed by the

translation of all the fabI gene sequences obtained in the previous step using a local run of Clustal Omega (Sievers and Higgins, 2018). The output sequences were then arranged in a single FASTA file. The resulting sequences were then viewed in JalView (Waterhouse et al., 2009), and the mutations were analyzed and compared against previously reported mutations from the fabI gene of microbes other than *K. pneumoniae*. For ease of view, colours were added based on the alignment score.

Statistical analyses

Experiments were performed on at least three independent occasions, and the resulting data are presented as the arithmetic mean of these biological repeats unless stated otherwise. Error bars, where shown, represent the standard deviation of the mean.

Materials availability statement

All materials created in this manuscript may be accessed by contacting the corresponding author, under appropriate material transfer agreements.

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Author contributions

Conceptualization and supervision: G.S.K. and M.S.; S.B. performed culture assay, synergistic, time-kill assay, S.G. carried out computational studies; S.D.P. performed Cell Viability and MTT Assay, P.M. performed biofilm and resistance of frequency assay; G.V. provided clinical

strain, N.M and M.S provided resources S.B. S.G., S.P., GSK wrote first draft of the manuscript; S.B., G.S.K., and M.S. finalized the manuscript with comments from all other authors.

Conflict of interest

KIIT University, Bhubaneswar, has filed a patent application with the Indian Patent Office for a combination formulation of polymyxin B and triclosan.

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