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Antimicrobial resistance and antimicrobial alternatives

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Antimicrobial resistance and antimicrobial alternatives

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Editorial: Antimicrobial resistance and antimicrobial alternatives

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Editorial on the Research Topic
Antimicrobial resistance and antimicrobial alternatives

Antimicrobial resistance (AMR) poses a relentlessly growing threat to human and animal health across the globe. The inappropriate use of antimicrobials in clinical and agricultural settings has created suitable conditions for the evolution, emergence, and dissemination of AMR. Overreliance on antimicrobials has resulted in an unprecedented selection pressure that spurred the proliferation of antimicrobial-resistant microorganisms in human, animal, and environmental microbiota (1). These conditions also facilitate the mobilization and horizontal transfer of resistance determinants from commensal bacteria to those that can cause disease; especially with the accumulation of evolutionary events that select for resistance in host populations and environmental ecosystems. Today, AMR is casting an ominous shadow; a critical health challenge with magnitudes dwarfing most life-threatening diseases such as HIV and malaria (2). It has been famously predicted that 10 million people could die annually by 2050 if robust interventions to control AMR were not adopted (2). Recently, a comprehensive assessment showed that AMR directly caused an estimated 1.27 million deaths and was associated with 4.95 million deaths globally (2). Taken together, AMR is a current and serious threat that might cause a global crisis, shifting the balance of the war against infectious diseases away from human and animal welfare. Unifying efforts to tackle the scourge of AMR are urgently required; including robust science-based monitoring of AMR transmission using the One Health ethos and investigating new advances in non-traditional and next-generation antimicrobial products that reduce reliance on conventional antimicrobials.

This Research Topic: "*Antimicrobial resistance and antimicrobial alternatives*" hosts 11 manuscripts, including seven original articles, one systematic review, two narrative reviews, and one perspective article. The manuscripts were contributed by 76 authors from

various institutions and research centers. This Research Topic is involved in addressing AMR challenges by assessing trends in antimicrobial use and the development of resistance, especially in developing countries, and exploring the antimicrobial properties of promising non-traditional products. Inconsistent antimicrobial stewardship programs and deficiencies in systemic surveillance of AMR in low- and middle-income countries (LMICs) have promoted the injudicious use of antimicrobials among humans and other animals. Consequently, multidrug-resistant (MDR) microorganisms have been steadily increasing (1). At the community level, Khan et al. showed that storage of antimicrobials is common in households in post-conflict regions in Pakistan, and the communities also suffer from a lack of awareness on antimicrobial use and AMR. At the hospital level, Sirilak et al. suggested the need for reducing the unnecessary use of antimicrobials by adopting the recommendations of the World Health Organization and revising local guidelines on the empirical treatment of postpartum infections in women with episiotomy lesions in Thailand, an upper-middle income country. Sirilak et al. reported no significant differences in postpartum infections between patients that received antimicrobial treatments in comparison to those who did not.

Although the highest toll of AMR burden is thought to affect developing countries (2), it is well-known that drug-resistant microorganisms can easily spill over across geographic borders to affect both developing and developed countries. Unfortunately, limited data have been published on the epidemiology of AMR in LMICs, particularly among vulnerable populations (e.g., refugees) and in non-clinical settings (e.g., community, environment). Moreover, currently available data are usually incomplete and might not provide a comprehensive representation of the AMR burden, because the findings are based on small-scale studies and are temporally and spatially limited due to the lack of resources. Therefore, it was very pertinent that Osman et al. argued that vulnerable populations, specifically refugees and their hosting communities in conflict zones, in the Middle East and beyond are at an elevated risk of life-threatening AMR infections. For example, Syrian refugees in makeshift camps and other disenfranchised populations in Lebanon are susceptible to infectious diseases and antimicrobial-resistant pathogens, which are amplified by COVID-19 and dire social and economic situations. In Lebanon, MDR Gram-negative bacterial infections have been reported in critical patients diagnosed with COVID-19 in clinical settings (Sleiman et al.). Interestingly, the mobile colistin resistance gene (*mcr-1.26*), previously isolated in a pigeon in Lebanon (3), was reported in a Lebanese hospital (Sleiman et al.). The spread of MDR isolates (including carbapenem- and third-generation cephalosporin-resistant strains) is of great concern, especially in LMICs (e.g., Lebanon) and the disenfranchised population that are experiencing compounded public health challenges. The findings corroborated those observed in China, which reported a rapid increase in the fecal carriage rate of extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* among healthy rural residents (Wang et al.). The most common AMR determinants documented in these residents were $bla_{\text{CTX-M-14}}$, followed by $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$, $bla_{\text{CTX-M-24}}$, and $bla_{\text{CTX-M-65}}$ (Wang et al.). To halt the dissemination of AMR in these socioeconomic settings, it is crucial to understand both the intrinsic and extrinsic factors that contribute to the emergence and persistence of resistance. Therefore, there is a paramount need for One Health-based surveillance studies that allow local and international stakeholders to improve AMR stewardship programs and address the calamitous problems that threaten these populations.

Given the rapid spread of resistance, it is imperative to seek novel antimicrobial agents and other therapeutic options to overcome increasing rates of AMR and treat life-threatening MDR infections. Eravacycline, a relatively new fluorocycline antimicrobial with broad-spectrum efficacy against common clinical pathogens, was not inferior to ertapenem and meropenem in adult patients with complicated intra-abdominal infections. Subsequently, Eravacycline might represent an excellent option for the treatment of these infections (Meng et al.). Although there is a pressing demand for new antimicrobials, the use of antimicrobial alternatives (i.e., non-antibiotic antimicrobial therapies) is also sorely needed, especially when noting the decline in the discovery and development of novel antimicrobial agents. It is no secret that pharmaceutical companies are reluctant to develop new drugs due to regulatory, scientific, and financial barriers (4). In this context, current efforts must expand toward promoting next-generation anti-infectives along with reducing the unnecessary use of antimicrobials. Vaccines and alternative therapies (i.e., bacteriophages, antimicrobial peptides, bacteriocins, lysins, CRISPR/Cas9, antibodies, antimicrobial adjuvants, probiotics, and microbiome alterations) must be explored and adopted as first-line options for infection prevention and treatment. For example, Blue light is emerging as a safe microbicidal tool in several clinical and other public health-associated applications, such as food safety, environmental decontamination, and treatment of clinically relevant pathogens (Haridas and Atreya). Furthermore, Gungordu Er et al. highlighted that graphene-based nanomaterials can be potential antiviral candidates for biomedical applications such as antimicrobial personal protective equipment. Future investigations must continue to explore more promising antimicrobial alternatives that can effectively control untreatable complicated infections, perhaps tackling etiologic agents in a variety of settings in the host and beyond. While progress is being made, the current challenge is to implement these alternative therapies in medical practices, prove their efficacy, safety, and affordability, and gradually replace/supplement conventional antimicrobial interventions.

In conclusion, a multi-pronged approach that engages all stakeholders (including scientists, legislators, clinicians, pharmaceutical companies, and the public) is necessary to avoid the tsunami of AMR. Resistance is not only a local challenge, but it also affects everyone across the globe and requires holistic consideration of clinical, agricultural, and environmental practices. Special and real support must be provided to protect the most vulnerable populations in locations where AMR can take hold, amplify, and spread; causing unimaginable suffering. The manuscripts published on this Research Topic highlighted the growing problems of inappropriate use of

antimicrobials and the spread of AMR and the need for effective antimicrobial alternatives.

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MO drafted and edited the manuscript. IA, KE, and IK edited the manuscript. All authors approved it for publication.

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Conflict of interest

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Catch-22: War, Refugees, COVID-19, and the Scourge of Antimicrobial Resistance

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Wars have hidden repercussions beyond the immediate losses of life, well-being, and prosperity. Those that flee wars and seek refuge in safer locations are not immune to the tragic impacts. Of particular concern is the susceptibility of the refugee populations to infectious diseases and antimicrobial-resistant pathogens. This poses a detrimental risk to these disenfranchised populations, who often have limited access to medical care, sanitation, and nutritious and safe food. Furthermore, antimicrobial-resistant pathogens in refugees can be both transmitted to and acquired from their hosting communities. The latter is particularly problematic when the host countries suffer from serious challenges such as limited resources, pollution, and widespread antimicrobial resistance (AMR). Here, we discuss AMR in refugees of the ongoing Syrian war, a conflict that resulted in the largest population displacement in recent history. We argue that Syrian refugees and their hosting communities are at an elevated risk of complicated and life-threatening AMR infections. We also call on the international community to address this grievous problem that threatens the disenfranchised refugee populations and can spill over across geographic borders to affect multiple countries.

Keywords: antimicrobial resistance, COVID-19, refugees, civil war, armed conflict, displaced populations, infectious diseases, one health

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INTRODUCTION

Multiple conflicts are currently unfolding across the globe. However, the protracted Syrian civil war resulted in the largest refugee crisis in recent history. Due to political and economic unrest and armed conflicts, Syrian civilians have been seeking refuge in different parts of the world. Of notable concern is the proliferation of infectious diseases that ruthlessly exploit the unprecedented humanitarian conditions created by the Syrian conflict (1–5). For example, refugees are at high risk of susceptibility and/or exposure to life-threatening infectious diseases such as COVID-19 (6, 7), tuberculosis (8), hepatitis A (1), measles (3), and poliomyelitis (4). In many instances, the

vulnerable and potentially immunocompromised refugees are exposed to conditions that favor the selection and acquisition of multidrug-resistant (MDR) infections, which also pose a heightened risk for a population with limited access to healthcare, immunizations, and essential medications (9–11).

Factors that favor the emergence of antimicrobial resistance (AMR) already existed in Syria before the conflict (11, 12). Although there are laws in Syria (number 2/T) that prohibit over-the-counter sale of antimicrobials and date back to 1988, Syria still experienced undeveloped and weakly enforced regulations and laws associated with the prudent use of antimicrobials (11). For example, concerns over losing clients were considered a primary driver that rendered antimicrobials readily accessible for purchase from pharmacies and agriculture drug stores without the need for a medical prescription (13). However, the ongoing Syrian conflict has created more favorable conditions for the selection and dissemination of AMR (11, 12). Indeed, existing reports on AMR in displaced Syrian populations have described increasing trends of MDR *Enterobacterales*, methicillin-resistant *Staphylococcus aureus* (MRSA), and drug-resistant tuberculosis (14–22). Taken together, it can be argued that there is an imminent threat to Syrian populations, especially refugees that live in camps with high exposure to pollution and limited access to water, sanitation, and hygiene (WASH) programs and adequate medical care. Additionally, the rise of AMR in Syrian populations has serious regional and global implications, because it can spill over to countries that host large populations of refugees.

A global health response is needed to implement comprehensive effective policies and intervention strategies to tackle the scourge of AMR in major locations and camps that host the refugees. However, this cannot be achieved without a deep understanding of the current epidemiology of AMR in these locations. Therefore, we aimed here to evaluate the threat of AMR in the context of the Syrian crisis. We also highlighted the humanitarian situation and AMR challenges that the Syrian refugees are facing inside Syria and in host countries, with a special focus on Lebanon, a country that is currently hosting ∼ 1.5 million refugees while facing its own challenges with high drug resistance trends (23–26) and calamitous economic and political crises. These conditions have resulted in a shortage of medicine, appropriate diagnostic tools, water, and other critical necessities in Lebanon (27, 28).

ANTIMICROBIAL RESISTANCE IN BRIEF: A GLOBAL PUBLIC HEALTH THREAT FACING HUMANITY

According to the World Health Organization (WHO), AMR is growing and has become one of the greatest public health challenges facing humanity (29, 30). In 2016, the Interagency Coordination Group on AMR predicted that drug-resistant infections could potentially cause up to 10 million deaths globally per year by 2050 (29). Perhaps predictably, the majority of the AMR-associated mortality and morbidity are projected to occur in countries with weak antimicrobial stewardship, low resources, and/or wide pollution. Furthermore, approximately five million deaths were associated with bacterial AMR in 2019; most of these cases included lower respiratory and bloodstream infections and a variety of etiologic agents such as *Escherichia coli*, *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* that exhibited resistance to multiple classes of critical antibiotics (31). These studies at least shed some light on the bummock of the AMR iceberg.

The problem of AMR is further complicated by a faltering research and development pipeline with only a few new drugs on the horizon. The latter is due to many factors, which are mainly related to pharmacoeconomics and the challenges, financial and otherwise, associated with discovering and bringing new antimicrobials to the market (32). For example, the cost of developing an antibiotic can be around 33-fold higher than the yearly revenue generated from the respective drug sales (33). Consequently, humanity is facing increasingly recalcitrant infections without enough new drugs to control resistant pathogens. This raises many questions, including how will disenfranchised populations (e.g., refugees) with limited resources and access to proper medical care cope? What is the toll on the hosting country, especially if it is a low- and middle-income country (LMIC)? The judicious answer, at least partially, is to preemptively control AMR infections in these populations and their role as reservoirs for the emergence and spread of AMR pathogens. To achieve this, robust surveillance and monitoring studies are needed to understand the drivers of AMR in these populations. That is not an easy task given the magnitude of displacement, the disparate locations, conditions of refugee camps, and lack of resources, among a plethora of other issues.

THE SYRIAN CONFLICT AND THE SCOPE OF DISPLACEMENT

Millions of Syrians have fled violence and persecution to seek safety as displaced individuals both inside and outside Syria. According to the current estimations of the United Nations High Commissioner for Refugees, more than two-thirds of the Syrian population have been internally displaced in Syria (∼ 6.7 million) and/or fled the country and sought asylum abroad (∼ 6.6 million), noting that there are large numbers of unregistered refugees that are not listed in the census (https://www.unhcr.org/en-us/syria-emergency.html). Furthermore, the Syrian crisis has, directly and indirectly, affected several other countries in the Middle East and Europe. Notably, Turkey has been hosting the highest number of Syrian refugees (3.75 million), followed by Lebanon (∼1.5 million), Jordan (0.67 million), Iraq (0.26 million), and Egypt (0.14 million). It is also estimated that one million Syrian asylum seekers and refugees have entered Europe, and the vast majority are hosted in Germany (59%) followed by Sweden (11%), while other European countries (Austria, Greece, the Netherlands, France, Kosovo, Serbia, Hungary, and Denmark) host between one to five percent.

Due to the massive influx of refugees, some European countries enforced laws and sanctions to prohibit refugees from seeking asylum on their lands. This along with the desperation to flee violence pushed the refugees to follow illegal and risky routes to enter Europe. The illicit entry caused a gap in the surveillance data and registry of Syrian families, further impeding them from accessing medical care, education, safe food, international funds, and humanitarian aid.

In many hosting countries, displaced Syrian populations live below the poverty line in makeshift tents and struggle to survive the elements and secure basic needs, depending largely on the support from the international community and the hosting countries. For instance, Syrian refugee households living in Lebanon are highly vulnerable and suffer from intense economic pressures (**Figure 1**). A large fraction of Syrian refugees continues to live in substandard residential (69%) and non-residential (11%) accommodations and informal tented settlements (20%) in Lebanon (34). In 2015, it was estimated that nearly 70% of Syrian refugee patients were forced to stop their medical treatments due to economic hardship (35). These deteriorating conditions have promoted the emergence of numerous public health problems, which overtaxed the fragile health systems in Lebanon. For example, the Lebanese National Tuberculosis Program (NTP) has shown a significant percentage of tuberculosis cases in Syrian refugees, leading to an increase in the incidences of tuberculosis in Lebanon (8). A nationwide study on the prevalence of tuberculosis in Lebanon described that approximately 30% of patients in the country were Syrians, and the Lebanese population only accounted for 28% of cases (22). Similarly, the Turkish Ministry of Health observed a noticeable increase in the percentage of imported tuberculosis cases, reaching approximately 7% in 2015 compared to 1.3% in 2011 (36). It is worth mentioning that the official data provided by the NTPs in Middle Eastern countries likely underestimate the actual rates of tuberculosis, particularly in areas experiencing economic and political collapse (11, 37). Taken together, these observations confirm that the prevailing conditions for large numbers of refugees are conducive for the acquisition of infectious diseases, especially in hosting countries that lack resources and face their own challenges.

ANTIMICROBIAL RESISTANCE AMONG SYRIAN REFUGEES: EVIDENCE FROM SEVERAL HOSTING COUNTRIES

Several reports have highlighted a high prevalence of drug-resistant etiologic agents among displaced Syrian populations and refugees and called for preemptive actions to tackle the problem. Notably, colistin, a last-resort antibiotic used to treat complicated infections, was effective on only 11% of the *P. aeruginosa* isolated from patients suffering from healthcare-associated infections in three major hospitals in Aleppo, Syria (38). Additionally, higher prevalence of MRSA and MDR *Enterobacterales* were found among Syrian refugees compared to the resident population in Germany, confirming previous findings from Finland, Denmark, Austria, Israel, Switzerland, and the Netherlands (11). Compared to German control populations, refugees from Syria showed large differences in the gut microbiota composition and an increased prevalence of AMR bacteria (39), where 6.3% and 1.6% of screened refugees carried MRSA and extended-spectrum cephalosporin-resistant *Enterobacterales* (ESCR-E), respectively (39). Another study in Germany found that patients admitted from refugee accommodations were notably more colonized with MRSA (10.3%) compared to German resident ICU patients without refugee history (1.4%) (40). Furthermore, 9.8% and 23.3% of the refugees exhibited colonization with MRSA and MDR Gram-negative bacteria in Frankfurt (Germany), respectively (41). MRSA is considered one of the top public health risks that affect minorities and immigrant populations and can cause invasive and life-threatening infections (42). Furthermore, resistance to extended spectrum cephalosporins (ESC) and carbapenems, which are also considered critically important antibiotics, is usually associated with resistance to other key antimicrobial agents. Losing the effectiveness of these antibiotic classes usually leads to more labor-intensive treatments and, in some cases, futile interventions (43).

It is important to note that the predominant proportion of new sensitive and resistant tuberculosis cases across the world are among immigrants, asylum seekers, and refugees (44). These populations have a heightened susceptibility to drug-resistant tuberculosis associated with worse clinical outcomes (21). For example, the first description of an extensively drug-resistant (XDR)-tuberculosis case in Lebanon was among refugees or asylum seekers from Syria, Sudan, and Ukraine (22). Although limited data have been published on the emergence and spread of MDR and XDR tuberculosis among Syrian refugees in host countries, drug-resistant isolates from Syrian refugees were reported in Lebanon (22), Jordan (45), Turkey (46), Germany (47), and the Netherlands (48, 49). Obviously, the transmission of MDR tuberculosis in these populations and during refugee migration (spread to hosting countries) poses a high public health priority for all stakeholders (50).

The spread of AMR in refugee populations is also evident in some of their ill-constructed camps and pollution-susceptible necessities such as domestic water. Studies have shown that the camp environment can be polluted and lead to an increased exposure of refugees to AMR pathogens and determinants (51, 52). The first data on the occurrence of colistin resistance and the mobile colistin resistance gene (*mcr-1*) in Syrian refugee camps were described in Lebanon, highlighting the detection of OXA-48 and KPC carbapenemases in some colistin-resistant isolates (53). Notably, the *mcr* gene was later found to be plasmid-borne, suggesting that it can transmit colistin resistance between bacterial species (54). Furthermore, genomic analysis of two of the *mcr* containing isolates showed the presence of an additional 14 to 19 AMR genes that encoded resistance to several classes of antibiotics, including aminoglycosides, diaminopyrimidines, macrolides, β-lactams, phenicols, fosfomycin, tetracyclines, fluoroquinolones, and sulfonamides (54). Colistin-resistant

FIGURE 1 | Photographic examples highlighting the conditions in refugee camps in Lebanon. White makeshift tents in proximity to hosting community residence (concrete building in the background) **(A)** and limited-capacity water cisterns **(A,B)** that provide domestic and drinking water to the camp inhabitants that include a large number of youths **(B,C)** and elderly individuals **(C)**. The cisterns are filled periodically via water trucks provided by non-governmental organizations (NGOs) and charitable donations. The source of the water varies, the supply is sporadic, and the cisterns are filled from underground cisterns/wells **(D,E)** when the trucked water supply is insufficient. The underground cisterns are very vulnerable (without proper protection) and prone to contamination from shallow and inappropriate camp sewage conduits **(F)**, especially after rainfall. The camp inhabitants have reported that the cisterns have been contaminated with sewage on multiple occasions. This spreads persistent contamination to drinking and domestic water cisterns. Camp environments are generally unclean (dust, stones, solid waste) as can be observed in all the pictures above. Photographs were documented by Mr. Abdallah AlHaj Sulaiman.

E. coli isolates from sewage and domestic water collected from the camps exhibited co-resistance to multiple other antimicrobials, including ESC (22–64%), carbapenems (6%), tetracycline (94%), chloramphenicol (94%), fluoroquinolones (75–86%), and trimethoprim-sulfamethoxazole (92%). In another study, molecular analysis of MDR *E. coli* isolated from sewage water in Syrian refugee camps located in the Beqaa Valley revealed that 53.1% of isolates were positive for numerous resistance determinants (such as CTX-M-14, OXA-1, SHV-12, CMY-2, aac(6)-Ib, acc(3)-II, and Int-I1) (55). The infrastructure in refugee camps is non-existent or weak at best, and makeshift sewage conduits in the camps were shallow and exposed. During heavy rains, these sewage outlets overflooded and leaked into domestic water cisterns, eventually contaminating drinking water. These studies were followed by investigations that reported for the first time the detection of *mcr-1* in *Proteus mirabilis* isolated from domestic and sewer water in Syrian refugee camps in Lebanon (56). *P. mirabilis* is an opportunistic pathogen that can cause a variety of illnesses, including complicated urinary tract infections that can lead to bacteremia. Taken together, these observations suggested that important AMR determinants may be circulating in different bacteria in the camp environment, and perhaps also being amplified in the refugees. The latter indicates a cycle of amplification and transmission between refugees and their camp environments. Notably, the untreated sewage generated from the camps in Lebanon ends up in nearby rivers or affects agricultural water channels (irrigation), posing a risk of transmission to the hosting community (57).

It should be noted that refugees are also exposed to AMR determinants circulating in the hosting country. This can be significant when considering the AMR trends in these countries. For example, in Lebanon, a recent report highlighted that the prevalence of *mcr-1*-positive MDR *E. coli* detected in Lebanese irrigation water was high (58). Also, the occurrence and persistence of transmissible *mcr-1* in colistin-resistant *E. coli* has been reported for the first time in the Mediterranean Sea along the coast of Lebanon (59). Additionally, another report showed that Lebanese rivers suffer from elevated fecal pollution and *E. coli* isolated from these waters exhibited resistance to ampicillin (40% of isolates), amoxicillin-clavulanic acid (42%), cefepime (4%), cefotaxime (14%), cefalexin (46%), cefixime (17%), doripenem (0.3%), imipenem (0.5%), gentamicin (6%), kanamycin (9%), streptomycin (35%), tetracycline (35%), ciprofloxacin (10%), norfloxacin (7%), trimethoprim-sulfamethoxazole (32%), and chloramphenicol (13%) (60). Of particular concern was that 45.8% of the isolates were MDR. These observations are significant, because many refugee camps use river water for domestic purposes, especially when water supplies (via donations or non-governmental organizations) to the camps are limited or unavailable. Furthermore, many camps are located in agricultural areas in close proximity to irrigation water, and many refugees seek recreation on freely-accessible public beaches and rivers in Lebanon.

Taken together, the susceptibility of refugee populations to AMR infections and their role in transmission of resistant pathogens across borders should not be viewed independently

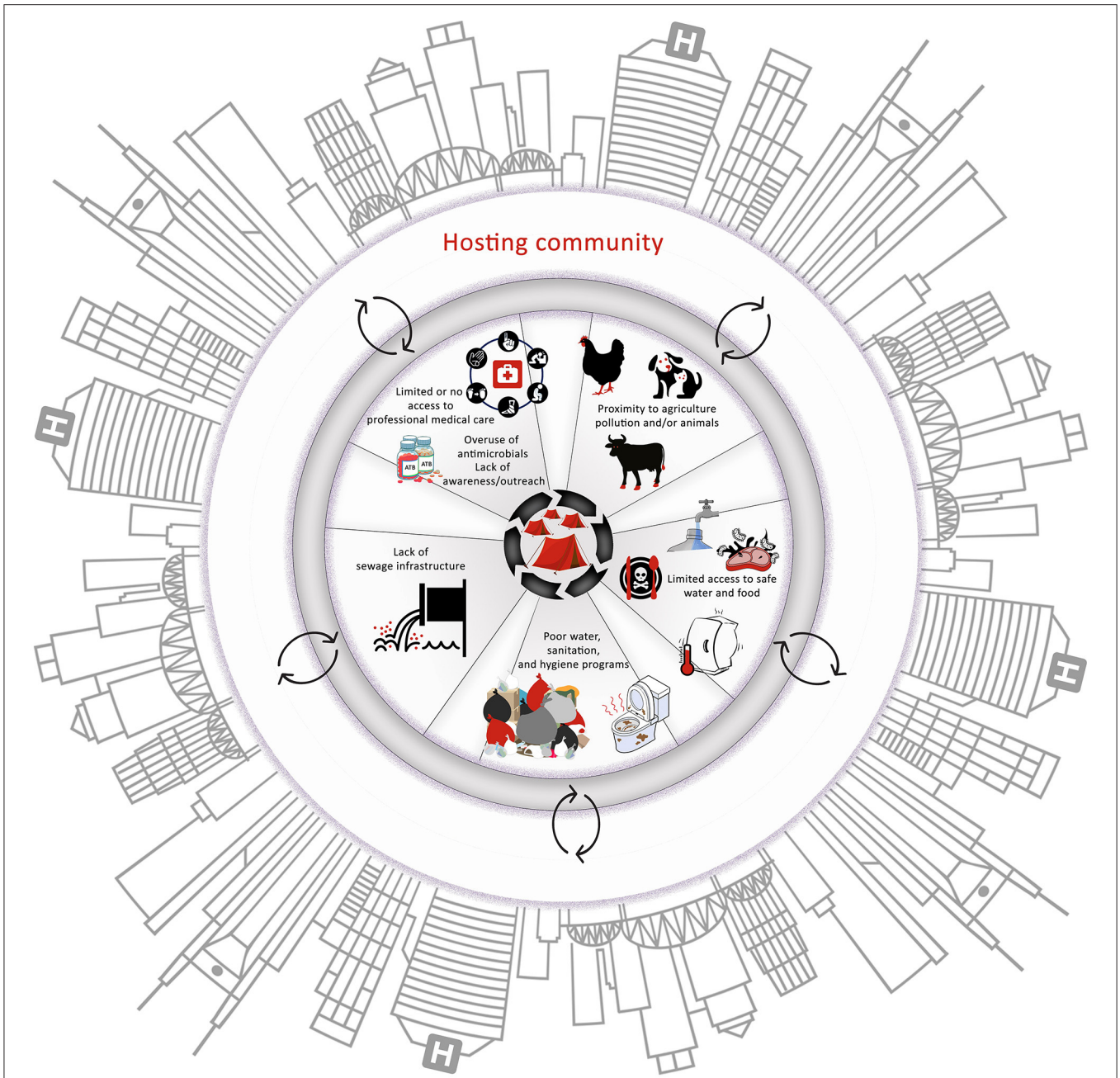

FIGURE 2 | The major factors that influence the emergence and spread of antimicrobial-resistant pathogens in the camps. It is important to note the interactions between all the factors and between the camps and the hosting community. These interactions can create suitable conditions for the emergence and rapid spread of antimicrobial resistance (AMR) within camps and hosting communities, highlighting the necessity to adopt a One Health approach to tackle AMR in these scenarios.

from the AMR trends in the hosting country. Again, the latter is especially true in LMICs that have widespread pollution and proliferation of AMR determinants. Stakeholders should be cognizant of the transmission dynamics of AMR in both refugees and the hosting countries (**Figure 2**); otherwise, interventions to control AMR in these populations might have a limited impact.

COVID-19, A FAILING ECONOMY, AND THE EMERGENCE OF INFECTIOUS DISEASES AND AMR IN SYRIAN REFUGEES: A FOCUS ON LEBANON

The healthcare system in Lebanon poses multiple challenges for refugees, including a lack of clarity, mistrust, discrimination,

and limited and fragmented services. Although health services are in principle available for registered and unregistered Syrian refugees in Lebanon for an affordable fee (61), 13% of patients had no access to any primary healthcare services and only 81% of patients seeking hospital care were able to receive the needed services (11). Approximately, two-thirds of the refugees have been reported to stop the use of medications due to the inability to afford medicines or handling fees (35). Notably, these challenges existed prior to the onset of COVID-19 and the collapse of the Lebanese economy.

Since October 2019, Lebanon has been witnessing an unprecedented economic collapse and inflation, which were further exacerbated by the emergence of the COVID-19 pandemic. Lebanon has experienced a large epidemic expansion (as of April 13, 2022, there were 1,094,911 cases as well as 10,350 COVID-19 deaths). Furthermore, the devaluation (> 90%) of the Lebanese pound has increased medical costs and pushed more than half of the resident populations, Lebanese and refugees, into extreme poverty. These populations have become unable to purchase personal protective equipment (e.g., masks, disinfecting products) or afford food and health services. Notably, Lebanon also relies heavily on imports to meet most of its food, energy, and medical needs (27). The inability to cover or even subsidize essential medications became an additional barrier to seeking healthcare for these vulnerable populations.

Given that the Lebanese government has immensely struggled to cope with the pandemic and provide care for Lebanese citizens, refugees were by default at heightened risk for SARS-CoV-2 infections. Weak public health interventions have been reported in refugee camps since the onset of the Lebanese economic crisis (62). United Nations data revealed that Syrian refugees have died from COVID-19 at a rate more than four times the national average (63). The living conditions of Syrian refugees in overcrowded shelters with scarce access to WASH and infection prevention and control (IPC) services likely allowed the occurrence of greater and more severe infectious disease outbreaks in high-density refugee camps, including SARS-CoV-2 outbreaks (57). Subsequently, to cope with challenges related to the lack of oxygen supply and limited healthcare, more antimicrobials were inappropriately used to treat COVID-19 patients (refugees and Lebanese patients). Indeed, anecdotal evidence suggested an overreliance on self-medication with azithromycin and other drugs in these populations. To date, there are no data on the scale of antimicrobial use in these settings during the pandemic. However, healthcare professionals have voiced great concerns about the inappropriate use of antimicrobials and unjustified prolonged antimicrobial treatments of patients infected with COVID-19 in Lebanon (64–67). Therefore, an increase in the burden of AMR has been predicted in Syrian refugees and other vulnerable populations in Lebanon as a result of both COVID-19 and the dire social and economic situation.

CONCLUSIONS

Displacement, dire living conditions, infectious disease, and AMR are real challenges that the Syrian refugees face on a daily basis. To prevent the exacerbation of these challenges and avoid catastrophic consequences on the refugees and their vulnerable hosting communities, engagement of stakeholders across the globe and investments in infrastructure and One Health approaches are critical. AMR surveillance systems and antimicrobial stewardship programs using genomic and machine-learning analyses are also needed to mitigate the sources of AMR affecting refugees and hosting communities and to optimize their access to necessary antimicrobials. International aid organizations must be cognizant of the transmission dynamics of AMR and play a key role in helping to implement reliable action plans to control AMR in both the refugees and hosting countries. The latter is critical in countries like Lebanon that suffer from limited resources and a variety of healthcare and economic challenges. It should be noted that the challenges discussed in this manuscript are not limited to Syrian refugees and can be experienced by other refugees such as the Rohingya, South Sudanese, and Congolese among others.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

MO and IIK contributed to the conception of the study and data acquisition and drafted the manuscript. IIK supervised the work. KC and KE critically revised the manuscript. All authors contributed to the article and approved the submitted version.

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The microbicidal potential of visible blue light in clinical medicine and public health

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Visible blue light of wavelengths in the 400–470 nm range has been observed to have microbicidal properties. A widely accepted hypothesis for the mechanism of microbial inactivation by visible blue light is that the light causes photoexcitation of either endogenous (present within the microbe) or, exogenous (present in the biological medium surrounding the microbe) photosensitizers such as porphyrins and flavins, which leads to the release of reactive oxygen species that subsequently manifests microbicidal activity. Some of the factors that have been observed to be associated with enhanced microbicidal action include increased duration of exposure, and either pre- or co-treatment with quinine hydrochloride. In case of bacteria, repetitive exposure to the blue light shows no significant evidence of resistance development. Additionally, visible blue light has exhibited the ability to inactivate fungal and viral pathogens and, multidrug-resistant bacteria as well as bacterial biofilms. Visible blue light has demonstrated efficacy in eliminating foodborne pathogens found on food surfaces and exposed surfaces in the food processing environment as well as in the decontamination of surfaces in the clinical environment to minimize the spread of nosocomial infections. We conclude from reviewing existing literature on the application of the blue light in clinical medicine and public health settings that this microbicidal light is emerging as a safer alternative to conventional ultraviolet light-based technologies in multiple settings. However, further comprehensive studies and thorough understanding of the mechanism of microbicidal action of this light in different scenarios is warranted to determine its place in human health and disease.

KEYWORDS

violet-blue light, antimicrobial, pathogen reduction, biofilm, microbes

Introduction

The purpose of this literature review is to provide a comprehensive overview of the potential of the blue light as a microbicidal tool by examining its clinical and public health applications as evidenced by experimental studies published in the last 10 years. Additional areas of investigation within this literature review will include the mechanism of pathogen inactivation by the blue light, conditions that optimize its microbicidal activity, its efficacy on different types and forms of pathogens, and its effects on normal healthy host (mammalian) cells.

The omnipresence of different forms of pathogens poses a multitude of risks to health of the individuals and population at large. For this reason, the technologies that reduce or inactivate pathogens have applications in food safety, environmental health, and clinical contexts. Emerging technologies for pathogen reduction can also have far-reaching public health consequences, with implications for long-standing issues like antibiotic-resistant bacteria, and the incidence of hospital-acquired infections. One such emerging technology is the application of visible blue light of wavelengths in the 400–470 nm range, hitherto referred as "the blue light" throughout this report, is increasingly being investigated for its microbicidal properties against a variety of pathogens in various settings (1, 2). Potential clinical applications of these technologies include the treatment of localized infections and wound healing (2, 3). The blue light also shows potential for facilitating a solution for the increasingly urgent public health concern of antibiotic resistance, as experiments involving repeated exposure to high intensities of blue light have shown no significant evidence of bacterial resistance (4–6). Environmental decontamination applications of the blue light include disinfection of food, contact surfaces, and surrounding air. Foodborne pathogens are estimated to cause annually 9.4 million cases of illness in the United States (7). Among the leading pathogens of these foodborne illnesses are norovirus and non-typhoidal *Salmonella* (7). Microbial persistence and growth on surfaces are another major health concern, particularly in the hospital settings (8). Dry hospital surfaces have been observed to develop biofilms of microbes, which are then less susceptible to conventional disinfection methods (8).

Mechanism of pathogen inactivation by the blue light

A widely accepted hypothesis regarding the mechanism of photoinactivation of organisms by the blue light is that it causes photoexcitation of endogenous photosensitizers such as flavins and porphyrins, which triggers the release of reactive oxygen species, and in turn leads to oxidative stress and cell toxicity; this was well described in Ramakrishnan et al. (9). This study made several observations using *Staphylococcus epidermidis* and 405 nm light as a model for photoinactivation of bacteria: (a) treatment resulted an overproduction of reactive oxygen species in the treated cells (b) upon addition of a reactive oxygen species (free H_2O_2) scavenger sodium pyruvate, to the light-treated *S. epidermidis* cells, the bacterial inactivation was substantially reduced when compared to light-treated *S. epidermidis* cells without sodium pyruvate, (c) greater reduction in *S. epidermidis* inactivation observed when a combination of three reactive oxygen species scavengers (sodium pyruvate, catalase, and dimethyl thiourea) was administered to light-treated cells. These observations lend credence to the fact that more than one reactive oxygen species takes part in the photoinactivation mechanism induced by 405 nm light (9).

A recent study investigated the reason for the known discrepancies in bacterial inactivation by different light wavelengths within the 400–470 nm spectra, with the understanding that differences in the extent of microbial inactivation are typically explained by the presence of different photosensitizers present within the targeted microbe (1). Investigators studied the apparent contradiction in existing knowledge which indicates that 400–420 nm light is more efficient at bacterial inactivation than 450–470 nm light, even for *Enterococci,* which possess flavins but not porphyrins that would predispose them to be more vulnerable to 400–420 nm light (1). Upon exposure of *E. moraviensis* to 405 and 450 nm light, it was found that 405 nm light exposure led to a greater reduction in the bacterial population (1), consistent with findings in prior publications. Fluorescent spectral analysis of *E. moraviensis* exposed to 405 nm light showed decreasing endogenous flavin levels with increasing duration, as well as increasing levels of hydrogenated nicotinamide adenine dinucleotide (NADH) and the fluorophore lumichrome in the bacteria (1). Based on this observation, investigators proposed that although both NADH and lumichrome were increased with increased light dose, the latter could possibly have a role in the efficient inactivation of *E. moraviensis* by 405 nm light, as it is known to have high absorption at 405 nm and is an efficient photosensitizer (1).

Another study elucidated the microbicidal mechanism of the blue light on *Neisseria gonorrhoeae* (3). Upon examination of the fluorescence spectra and absorption spectra of endogenous photosensitizers derived from the *N. gonorrhoeae* cell lysates, presence of both porphyrins and flavins were identified; spectroscopic and ultra-performance liquid chromatography analysis showed that exposure to 405 nm light led to the excitation of porphyrins and flavins and subsequent cell death (3). This study also examined the microbicidal effect of 405 nm light under the hypoxic condition by deoxygenating the *N. gonorrhoeae* cell suspension with N2 prior to the light exposure. It was found that the microbicidal effect of 405 nm was substantially diminished in the hypoxic condition compared

to the non-hypoxic control, with only 1-$\log_{10}$ reduction and a complete inactivation of the microbe in the non-hypoxic control condition; the fact that there was still some level of bacterial reduction in the hypoxic condition led investigators to infer the involvement of reactive oxygen species (ROS) besides singlet molecular oxygen ($1O_2$) in the photoinactivation mechanism, which in turn suggests that flavins that produce ROS have a role in the photosensitization reaction and subsequent photoinactivation of *N. gonorrhoeae* by 405 nm light (3).

Conditions that optimize microbicidal activity of the blue light

A common finding among studies examining photoinactivation of bacteria upon administration of the blue light is that 405-nm light is the most effective at bacterial reduction, compared to other wavelengths within the 400–470 nm range (1, 3, 10). A study comparing the efficacy of photoinactivation of *Listeria monocytogenes* by blue light wavelengths ranging from 400 to 450-nm in 10-nm increments observed 405 ± 5 nm to be the most efficacious (10). As previously mentioned, it was found in the study by Hessling et al. sthat administration of 405 nm light led to a greater reduction in the bacterial concentration of *Enterococus moraviensis* than did administration of 450 nm light (1). Similarly, Wang et al. study examining the role of wavelength on inactivation of *Neisseria gonorrhoeae* found that 405-nm light led to greater reduction in bacterial concentration than did 470 nm light (3).

McKenzie et al. used 405 nm light in conjunction with different stress conditions to identify the stress conditions that most enhance the bactericidal activity of 405 nm light (11); in these experiments *Escherichia coli* and *Listeria monocytogenes* were subjected to sub-lethal stress conditions including varying temperatures, pH levels, and salt concentrations. After being held in the sub-lethal stress conditions, the bacterial suspensions were exposed to 405 nm light at an irradiance of 70 mW/cm^2 (11). Temperature stress was observed to induce enhanced photoinactivation with 405-nm light in both species, with the populations grown in the 4° and 45°C stress conditions exhibiting greater inactivation rates than those grown in the non-stress 22°C condition (11). Acid stress was observed to be associated with the most substantial enhancement of photoinactivation (11). After being held in the pH 3 condition, the dose of 405 nm light required to achieve the same extent of inactivation as observed in the non-stressed population was reduced by 77% for *E. coli* and by 50% for *L. monocytogenes* (11). Increased salt concentration produced differential effects on the two species, with *E. coli* requiring a 50% lesser dose for inactivation, whereas *L. monocytogenes* required a 50% higher dose for inactivation (11). Upon further examination, the apparent increase in 405 nm light tolerance in *L. monocytogenes* was explained by the fact that the increased salt condition did not induce sub-lethal damage in *L. monocytogenes* as it did for *E. coli*, as *L. monocytogenes* is known to have a degree of osmo-tolerance (11). Bache et al. comparing the extent of bacterial reduction after different durations of visible blue light treatment, it was found that increased duration of exposure was associated with greater extent of bacterial reduction (12).

Bacteria do not develop resistance and drug-resistant bacteria show susceptibility to the blue light

Drug-resistance in bacteria is an increasingly important issue that poses a substantial threat to public health on the global scale, as multidrug-resistant bacteria continue to emerge (13, 14). Infectious agents are a major contributor to the global burden of disease and are associated with more than 25% of annual deaths worldwide (13, 15). The development of drug resistance in bacteria limits the prevention and treatment options, and it is therefore of significant consequence to determine both the potential for bacteria to develop resistance to inactivation by the blue light, and the efficacy with which the light can inactivate drug-resistant bacteria. Multiple studies sought to determine whether bacteria are likely to develop resistance to photoinactivation by the blue light and observed no signs of the development of resistance in bacterial samples subjected to repetitive exposures of sublethal light doses (4–6, 16, 17). A 2019 study assessed whether *N. gonorrhoeae* cells could develop resistance to the 405 nm blue light by exposing the bacterial suspension to a subtherapeutic amount of the light, growing the surviving cells in culture overnight, exposing the new suspension to 405 nm light, and repeating this cycle for 15 times (6). Analysis of the bacterial reduction after each successive round of 405 nm light exposure showed no statistically significant association between the number of exposures and extent of bacterial reduction, suggesting that *N. gonorrhoeae* is not inclined to develop resistance to 405 nm antimicrobial blue light (6).

A 2017 study reached a similar conclusion after examining the propensity of *Staphylococcus aureus* to develop resistance to 405 nm light exposure (5). Tomb et al. study conducted one experiment in which *S. aureus* was cultured under low-level exposure to 405 nm light, at an irradiance level of 1 mW/cm^2 (5). In the second experiment, *S. aureus* was subjected to 15 successive cycles of exposure to a sublethal dose of 108 J/cm^2 of high-intensity 405 nm light (5). The *S. aureus* cultured under low-level light exposure was observed to require a greater dose of 405 nm light for complete inactivation (5). Upon the subsequent observation of elevated H_2O_2 in the

S. aureus cultured in low-level 405 nm light, it was suggested that the apparent tolerance of these cells to inactivation by 405 nm light may be attributable to the upregulation of protective enzymes induced by the stress of low-level 405 nm light exposure during culture (5). This finding suggests that to avoid engendering bacterial tolerance to 405 nm light in clinical applications, 405 nm light should be administered at a bactericidal dose as opposed to a low-level exposure for longer times (5). In the experiment subjecting *S. aureus* to 15 successive cycles of high-intensity 405 nm light at a sub-lethal dose, there was no significant evidence of the development of tolerance to inactivation by 405 nm light (5). The observation that bacteria do not appear to develop resistance to blue light after repetitive exposure was also supported by the findings of Zhang et al. which subjected multidrug-resistant *A. baumannii* to 10 successive cycles of sublethal exposure to 415 nm light and observed no development of resistance (17). Leanse et al. concluded that there was no statistically significant correlation between the number of exposure cycles to 405 nm light and the susceptibility to photoinactivation *in vitro* for any of the three gram-negative bacteria examined, *P. aeruginosa*, *A. baumannii*, or *E. coli* (16).

The effect of the blue light on drug-resistant strains of bacteria is also of significant consequence, as the growing issue of antibiotic-resistant bacteria presents a need for novel methods of controlling bacterial spread (18). Multiple studies have shown the blue light to have efficacy in inactivating multidrug-resistant bacteria (18–22). In a 2010 study that administered high-intensity narrow-spectrum light (HINS-light) with a peak wavelength of 405 nm on a hospital isolation room with high levels of *S. aureus* and methicillin-resistant *S. aureus* (MRSA), the use of HINS-light was associated with a reduction from 0.84 colony forming units/square centimeter (CFU/cm^2) to 0.42 CFU/cm^2 in *S. aureus* count, and a reduction from 0.73 to 0.26 CFU/cm^2 in MRSA count (22). Barneck et al. found 405-nm light to be effective in inactivating *E. coli* that had been conferred with ampicillin resistance, with an 82% mean reduction after 120 min of exposure at an irradiance of 2.89 mW/cm^2, and a 100% mean reduction after 120 min of exposure at an irradiance of 9.37 mW/cm^2 (19). Bumah et al. observed antimicrobial blue light of wavelengths 405 and 470 nm to be effective in reducing MRSA (20). A trend seen in this study was that greater fluences of light were required to achieve 100% reduction in the more dense (greater CFU/mL) bacterial colonies (20). In an *in vitro* study, suspensions of multidrug-resistant strains of *E. coli* were seen to be susceptible to photoinactivation by 410 nm light, with all strains exhibiting a greater than 3-log$_{10}$ reduction after 180 min of the light exposure (21). The strains of *E. coli* used in this study were resistant to colistin, broad spectrum cephalosporin, and carbapenem antibiotics that are considered the last resort for controlling multidrug-resistant bacteria (21). Leanse et al. proposed that antimicrobial blue light could be used in conjunction with quinine hydrochloride to inactivate multi-drug resistant bacteria (18). The use of quinine hydrochloride in conjunction with 405 nm light irradiation was associated with a greater than 10^3-fold reduction in planktonic suspensions of multidrug-resistant strains of *P. aeruginosa* and *A. baumanii* (18). It has also been proposed that sublethal doses of visible blue light can be used in conjunction with traditional antibiotics to treat multidrug-resistant bacteria (23). The findings of Wozniak et al. showed extensively drug-resistant *A. baumannii* to have enhanced susceptibility to antibiotics after sublethal exposure to the antimicrobial blue light (23).

There is consensus among studies that there is a difference in susceptibility to 405-nm light between gram-positive and gram-negative bacteria, with gram-positive bacteria typically being the more vulnerable and gram-negative bacteria requiring relatively greater doses of 405-nm light for inactivation (11, 24). McKenzie et al. study found that *L. monocytogenes*, a gram-positive bacterium, had greater susceptibility to inactivation by 405 nm light compared to *E. coli*, a gram-negative bacterium (11). After culture in optimal growth conditions, complete inactivation of 5-log$_{10}$ populations was achieved with a dose of 84 J/cm^2 405 nm light for *L. monocytogenes*, whereas a greater dose of 378 J/cm^2 was required to achieve the same extent of inactivation of the *E. coli* populations (11). A contrasting finding was observed in a 2016 study which examined the relative bactericidal effects of 405 nm light against four different clinically relevant bacteria and found gram-positive and gram-negative bacteria to have similar susceptibility to 405 nm light, with no statistically significant differences in susceptibility between the species tested (19). This study found ampicillin-resistant *E. coli* to be the most susceptible to photoinactivation by 405 nm light, followed by *S. pneumoniae*, *S. aureus*, and *P. aeruginosa* (19). This departure from the more widely observed tendency of gram-positive bacteria to have greater susceptibility to 405 nm light than gram-negative bacteria was attributed to possible variability in species, strains, and stain family (19). Since Gram-positive bacteria have a thick peptidoglycan layer and no outer lipid membrane whilst Gram-negative bacteria have a thin peptidoglycan layer and have an outer lipid membrane, the observed differential responses of the bacteria to the blue light could potentially be attributable to the differences between the cell membranes of Gram-positive and Gram-negative bacteria. Further investigations in this area are warranted.

Fungal inactivation potential of the blue light

The blue light has demonstrated efficacy across several studies in inactivating fungal pathogens. Studies have shown the blue light to be effective in inactivating *Candida albicans*, a clinically relevant fungal pathogen responsible for commonly

seen fungal infections (2, 25). It has been proposed that endogenous photosensitizers may be responsible for the phototoxicity effect of antimicrobial blue light on *C. albicans*, as fluorescence spectroscopy has indicated the presence of porphyrins and flavins in *C. albicans* (2). One such study administered 405 nm light to *ex vivo* rabbit corneas with *C. albicans* keratitis and concluded that it was indeed an effective antifungal treatment and a viable option for treatment pending studies investigating its safety for the surrounding eye tissue (25). *In vitro* experiments conducted by Zhang et al. exposing both human keratinocytes and *C. albicans* to 415 nm light showed that *C. albicans* was more susceptible than human keratinocytes to inactivation upon light exposure, and that there is a therapeutic window of exposure wherein *C. albicans* cells will be inactivated and human keratinocytes unharmed (2). In the *in vivo* experiment by Zhang et al., administering 415 nm light to *C. albicans*-infected mouse burn wounds, the light exposure substantially reduced the fungal burden, although there was a recurrence of infection following the cessation of light treatment (2).

In a study using *Saccharomyces cerevisiae* as the model organism, it was found that a one $\log_{10}$ reduction of the pathogen could be achieved with a 182 J/cm^2 dose of 405 nm light, and with a 526 J/cm^2 dose of 450 nm light (26). Subsequent analysis of trypan blue staining produced no evidence of damage to the cell membranes of the light-treated samples, suggesting that the visible blue light treatment did not impair the viability of the fungal cells but rather impacted their ability to be cultured (26). Analysis of the *S. cerevisiae* cell lysates indicated that the presence of the endogenous photosensitizer protoporphyrin IX may play a role in the sensitivity of the fungal cells to 405 nm light (26). The sensitivity of the fungal cells to 450 nm could conceivably be explained by the presence of riboflavin and other flavins (26).

An area for future investigation within the application of antimicrobial blue light to fungal pathogens is the impact of melanization (2). Fungal pathogens like *C. albicans* have been known to produce melanin in the context of an infection, which could conceivably shield fungal cells from light exposure and thus impact the efficacy of fungal inactivation by antimicrobial blue light (2). Future studies investigating the interaction between this phenomenon and antimicrobial blue light will be necessary to more fully ascertain whether antimicrobial blue light is a viable disinfecting strategy for fungal pathogens in public health and clinical applications.

Viral inactivation potential of the blue light

Because virions are known not to contain endogenous porphyrins, there is relatively less understanding of the mechanism and efficacy of antimicrobial blue light in inactivation of viruses compared to bacteria and fungi (27). There have been studies investigating the potential of the blue light for inactivating human norovirus, which is a leading cause of acute gastroenteritis and can be transmitted in a variety of ways including contaminated food and surfaces (27, 28). A major limitation of studies that seek to investigate the inactivation of norovirus, an issue of substantial clinical and public health importance, is that there is currently no standardized protocol for propagation of human norovirus, and experimental studies must be conducted using a surrogate virus (27, 28). Recently for the first time, noroviruses were shown to replicate in stem-cell derived human enteroids that would pave the way for a more systematic *ex vivo* culture of this group of viruses in the long-term (29).

A 2017 study sought to investigate the potential of the blue light as a preventative tool for norovirus outbreak in hospital settings, using Feline Calicivirus (FCV) as a surrogate for human norovirus (27). Female embryonic cells were inoculated with FCV and incubated, and after 90% of the cell monolayer was destroyed, the virus-containing supernatant was collected and stored (27). The media in which the virus-containing supernatant was suspended during exposure to the 405 nm light treatment appeared to affect the efficiency and extent of virus inactivation, with minimal media requiring the greatest dose, presumably because of the absence of porphyrins both in the virus and in the medium (27).

It was seen that FCV suspended in organically rich media or biologically relevant organically rich media showed the greatest reductions in FCV and necessitated relatively lower doses of 405-nm light (27). These findings support the basic principle that the presence of porphyrins enhances inactivation by 405 nm light, as the organically rich and biologically relevant media contained porphyrins, whereas minimal media did not contain porphyrins and showed relatively lower efficiency in FCV inactivation (27). The greater susceptibility of FCV when in the presence of biologically relevant substances including plasma and artificial saliva suggest that 405 nm light could potentially be a promising microbicidal tool in the contexts, where virus contaminants are likely to be in the presence of biological media containing endogenous photosensitizers.

Another study which used Tulane virus as a surrogate for human norovirus in the context of food safety and the decontamination of the produce also found that viral inactivation by 405 nm light was limited in the absence of some form of enhancer (28). Although the singlet oxygen enhancers riboflavin and rose Bengal did appear to enhance viral inactivation by 405 nm light, the overall extent of inactivation was limited, and there is a need for further research into food-grade compounds that can more substantially enhance the production of reactive oxygen species and subsequently inactivate viruses (28) while the basic mechanism of 405 nm light inactivation of microbes is

implicated through the production of radical oxygen species by the excitation of porphyrins or flavins present either within the organism or in the surrounding environment as suggested in section Mechanism of pathogen inactivation by the blue light of this review.

The blue light potential in inactivation of biofilms including drug-resistant biofilms

Biofilms are aggregations of microorganisms that are understood to be an adaptive mechanism to improve survival under conditions of stress (29). Biofilms have been observed to form on both living and non-living surfaces in clinical settings, and their increased resistance to antimicrobial treatments and host immune response present a considerable risk for the spread of infectious diseases (29, 30). The increased resistance to stress conditions observed in bacterial biofilms is thought to be attributable to the formation of an extracellular polymeric substance (EPS) that envelops bacteria (29, 31, 32). Polymicrobial biofilms, biofilms composed of more than one species of microorganism, are thought to have increased antimicrobial resistance attributable to the transfer of antimicrobial resistance genes between microorganisms (33). Studies have investigated the use of the blue light as an microbicidal strategy targeting both monomicrobial and polymicrobial biofilms.

The blue light wavelengths have been observed to inactivate bacterial biofilms, as seen in a 2020 study which concluded that 400 nm light effectively inactivated *Pseudomonas fluorescens* and *S. epidermidis* biofilms (34). Ferrer-Espada et al. concluded that 405 nm light exhibits efficacy in inactivating monomicrobial biofilms, with the *A. baumannii*, *S. aureus*, *N. gonorrhoeae*, and *P. aeruginosa* biofilms being the most susceptible to inactivation (31). It has also been shown that the monomicrobial biofilms composed of multidrug-resistant strains of *A. baumanni* and *P. aeruginosa* could also be inactivated with the 405 nm light treatment (31). In a study that applied 405-nm visible blue light to monomicrobial and polymicrobial biofilms, significant bacterial reductions were observed after light treatment (33). Monomicrobial biofilms of *P. aeruginosa*, *C. albicans*, and methicillin-resistant *S. aureus* were subjected to 405 nm light at a dose of 500 J/cm^2, and the resultant inactivation in CFU were 6.30log$_{10}$, 2.33log$_{10}$, and 3.48log$_{10}$, respectively (33). The polymicrobial biofilms composed of *P. aeruginosa* and *C. albicans* exhibited a 6.34log$_{10}$ inactivation in *P. aeruginosa* and a 3.11log$_{10}$ CFU inactivation in *C. albicans* after exposure to the same 500 J/cm^2 dose of 405 nm light (33). In the polymicrobial biofilms composed of *P. aeruginosa* and methicillin-resistant *S. aureus*, the same 500 J/cm^2 dose of 405 nm light led to a 3.40log$_{10}$ CFU inactivation of *P. aeruginosa* and a 2.37log$_{10}$ CFU inactivation of methicillin-resistant *S. aureus* (33). This study demonstrated the efficacy of 405 nm blue light against polymicrobial biofilms and suggests the importance of future *in vivo* studies to determine whether this could be applied to the clinical setting in the context of wound healing (33).

A study by Liu et al. applied 405 nm light to biofilms of *Moraxella catarrhalis*, a common causative pathogen for otitis media seen in children which is also known to form biofilms in the middle ear (32). Upon exposure to 405 nm light at a dose of 216 J/cm^2, a reduction in biofilms of greater than 3log$_{10}$ CFU was observed (32). Scanning electron microscopy analysis of the light-treated *M. catarrhalis* biofilms showed evidence of damage to the pathogen cell walls and loss of the EPS which provides structure to the biofilms (32). The findings of this study suggest a potential for 405 nm light to be used as a treatment for *M. catarrhalis* associated otitis media, pending further investigation to determine a suitable mode of delivery of 405 nm light to the middle ear in the clinical setting (32).

A 2020 study investigated the effect of visible blue light on *Pseudomonas aeruginosa* biofilms, which pose a significant risk of nosocomial infection in the clinical environment (35). It was observed that 410 nm light at a low dose of 75 J/cm^2 effectively prevented the formation of the biofilm matrix structure, and the higher doses of 410 nm light at 225 and 450 J/cm^2 were observed to cause damage to the bacterial cells themselves and impair further cell growth (35). The elimination of existing biofilms by 410 nm light was observed to be dose-dependent, with greater doses of light being associated with greater efficacy in decreasing the biofilm matrix, detaching the bacterial cells, and inactivating the bacterial cells (35).

The blue light potential in food safety from pathogens

Foodborne illness is a major public health concern in the United States, and proper prevention of these illnesses necessitate the use of effective disinfection techniques to ensure the safety of the food supply (24). Existing decontamination methods including thermal control, ultraviolet light, and chemical sanitizers have raised concerns about reduced nutritional value and carcinogenicity of the food product (24). Ultraviolet light has been demonstrated to be efficacious for decontamination in the food industry in contexts such as juice pasteurization and meat treatment, although its viability for widespread use in the food industry is limited by factors including safety concerns, limited ability to penetrate through packaging material, and potential for degradation of plastics (11, 36). A 2016 study examined the viability of the blue light treatment as a food disinfection technique by administering different doses of the light to processed meat

inoculated with *E. coli* and to cucumbers inoculated with *Salmonella* Typhimurium (24). In both treatment conditions, there was an observed increase in inactivation rate of the bacteria with increasing fluence, with complete inactivation of the *S.* Typhimurium on cucumber peels with administration of 464 nm light at 18 J/cm^2, and 96.3% inactivation of *E. coli* on processed meat with administration of 405 nm light at 100 J/cm^2 (24). While this study establishes that antimicrobial blue light is effective against *E. coli* and *S.* Typhimurium present on the food surfaces, it remains to be seen whether the blue light has any side effects on the quality and safety of the light-treated food items themselves (24).

A McKenzie et al. study subjected *E. coli* and *L. monocytogenes* to sub-lethal stress conditions that mimic the conditions that bacterial contaminants would likely be exposed to in the food services environment, including hot and cold temperatures, acidic environments, and high salt concentration environments (11). This study found that with both *E. coli* and *L. monocytogenes,* bacterial populations that experienced sublethal damage were more susceptible to inactivation by 405 nm light compared to bacterial populations grown in optimal conditions (11). Investigators propose that the observed enhancement in the bactericidal effects of 405 nm light when applied in conjunction with sub-lethal stress suggests a potential application of 405 nm light for decontamination of the food processing environment and reduction of the spread of foodborne pathogens (11).

Human norovirus is another major cause of foodborne illness, and a 2017 study sought to evaluate 405 nm light as a potential preventative tool by examining its effects on Tulane virus (a human norovirus surrogate) present on the surface of blueberries (28). Because viruses do not contain porphyrin, a major photosensitizer involved in the inactivation of bacteria and fungi by antimicrobial blue light, investigators in this study added food-grade singlet oxygen enhancers to the blueberries to enhance the production of the reactive oxygen species and thereby enhance the inactivating effect of the 405 nm light (28). Blueberries were inoculated with the Tulane virus, treated with singlet oxygen enhancers, and subjected to 30 min of 405 nm light exposure (28). In blueberries prepared with the singlet oxygen enhancer riboflavin, the 405 nm light treatment group exhibited greater inactivation of Tulane virus, with a statistically significant difference between the treated and untreated group (28). Among blueberries prepared with the singlet oxygen enhancer rose Bengal, the 405 nm light-treated group showed greater inactivation of the virus, although the difference between the treated and untreated group was not statistically significant (28). Investigators concluded that there is a need for further research of singlet oxygen enhancers that can be used in conjunction with 405-nm light to inactivate a virus more substantially like human norovirus and help ensure the decontamination of produce (28).

The blue light potential in decontaminating surfaces and environment

Nosocomial infections, also referred to as healthcare-associated infections (HAI) are a cause for increased morbidity and mortality of hospital patients, and their prevention necessitates safe and effective methods of environmental decontamination within the hospital (37, 38). Nosocomial infections are also associated with prolonged hospital stay and increased healthcare costs, and new technologies for the decontamination of surfaces and equipment could potentially help to reduce the burden of disease attributable to nosocomial infection (38). The blue light has been investigated as a tool for environmental decontamination, as exemplified by the high-intensity narrow-spectrum light environmental decontamination system (HINS-light EDS) which uses a peak wavelength of 405 nm and has been shown across studies to have efficacy with inactivating environmental pathogens (22, 37, 39).

Burns patients are particularly susceptible to infection due to the immunosuppressed state and impaired integrity of the skin, and the complications can include sepsis and organ failure (12, 37, 40). Burns patients are also likely to disperse bacteria into the surrounding environment, which makes infection control practices in burns units vitally important (12, 37). It has been observed that infection control practices in burn units have helped reduce fatality rates of burns patients (41). Maclean et al. examined the efficacy of 405 nm light for disinfecting a hospital isolation room in the hospital burns unit (22). Two such light sources were fitted in the ceiling of the hospital isolation room, and bacterial growth was examined in an unoccupied room, in an occupied room with intermittent 405 nm light application, in an occupied room with constant 405 nm light application, and in an occupied room without 405 nm light (22). Bacteria were sampled from frequently touched surfaces in the isolation room and examined after a 48-h incubation period (22). Results showed that the 405 nm light effected a statistically significant reduction in bacterial counts in each experimental condition (22). It was also noted that bacterial counts remained low for a period after discontinuation of the light application in the unoccupied room, in contrast to the occupied room which exhibited an increase in bacterial contamination up to pre-treatment levels within 2 days of the discontinuation of 405 nm light treatment (22). As this study administered the 405 nm light treatment concurrently with existing hospital decontamination practices, it was demonstrated that the 405-nm light treatment successfully achieved substantial bacterial decontamination that exceeds those achieved by currently used hospital decontamination practices alone, and thus provides strong evidence in support of the application of 405-nm light as a supplementary hospital decontamination method in the setting of a burn unit hospital environment (22).

Another study applied 405 nm light EDS to an inpatient burn isolation room occupied by a single burns patient and to an outpatient burn clinic room with 7–12 burns patients (37). As with the 2010 Maclean et al. study, samples were collected from several frequently touched surfaces and examined after a 48-h incubation period (37). With 14-h treatment of HINS-light for two consecutive days, a 27–75% reduction in bacterial CFU was observed from samples collected at 08:00 h from the inpatient burn isolation rooms (37). In the study conducted on the outpatient burn clinic rooms in which multiple patients were treated for burn wounds, the 8-h application of 405 nm light was associated with a lesser increase in bacterial contamination after the clinic closed; the outpatient room without 405 nm light exhibited a mean increase of 14.1 CFU/plate during clinic operation, whereas the outpatient room with 8-h 405 nm light treatment exhibited a mean increase of only 5.5 CFU/plate during clinic (37). Although complete bacterial inactivation was not achieved, this study provides further evidence of the efficacy of violet blue light in its potential application as a supplementary environmental decontamination tool in the inpatient and outpatient burn treatment settings to help prevent further spread of nosocomial infections (37). The results of the outpatient clinic experiment indicated that the 405-nm light treatment may be a promising decontamination tool for communal environments where cross-contamination by multiple individuals may be a concern (37). Bache et al. also applied 405 nm light treatment to a hospital burns unit at different irradiance levels and for different durations of exposure (12). While no correlation was observed between irradiance level and bacterial reduction, it was observed that increased duration of exposure was correlated with increased reduction in bacteria (12). As in the 2010 study by Maclean et al. and the 2011 study by Bache et al. the 2017 study demonstrated a decrease in bacterial counts upon exposure to 405 nm light treatment, and an increase in bacterial counts following the discontinuation of 405 nm light treatment (12).

Maclean et al. tested the 405-nm light technology in an intensive care unit (ICU) (39). An ICU room occupied by a patient for 9 days prior to the start of the study exhibited a 67% mean percentage reduction in *staphylococcal* counts following 405 nm light treatment (39). A second experiment with a patient who had arrived at the ICU room just 12 h prior to the start of the study did not exhibit a statistically significant mean percentage reduction in *staphylococcal* counts after 405 nm light exposure, and this was thought to be due to a lower starting level of bacterial contamination as the room had been cleaned more recently than in the previous experiment (39). Samples collected 24 h following the discontinuation of 405 nm light exposure exhibited a 357% increase in *staphylococcal* count, which supports the hypothesis that the 405 nm light has a substantial decontaminating effect (39). In a third experiment, the effect of direct vs. indirect exposure to the 405 nm light was examined (39). The sampled surfaces under direct exposure were observed to have a 63% mean reduction in bacterial counts associated with HINS-light treatment, and a 94% mean increase after discontinuation of the 405 nm light (39). The sampled surfaces under indirect exposure to 405 nm light were observed to have a 48% mean reduction in bacterial counts, and a 71% mean increase after discontinuation of 405 nm light (39). These results showed that both direct and indirect exposure to 405 nm light is associated with a significant reduction in bacterial contamination, although direct exposure does lead to a greater extent of bacterial inactivation (39).

A 2017 study applied visible blue light to the issue of indoor air pollution, and specifically aimed to degrade gaseous formaldehyde which can be injurious to health (42). Investigators first synthesized a titanium dioxide photocatalyst doped with the elements W, Ag, N, and F such that it would have photocatalytic activity in the visible light wavelengths, then combined the photocatalyst with formaldehyde in test tube reactors which were then exposed to visible blue light (42). Under this experimental setup, it was observed that the combined multi-element doped titanium dioxide was the most effective catalyst with 88.1% photocatalytic degradation of gaseous formaldehyde (42). The findings of this study suggest a potentially promising application of visible blue light in conjunction with photocatalysts for the purification of air (42).

The blue light potential in clinical applications to control localized infections without harm to human cells

Multiple studies examining the viability of the blue light for pathogen inactivation in clinical contexts have observed that photoinactivation of clinically relevant pathogens can be achieved at lower dosages of the blue light relative to the dosages required to cause damage to human cells (2, 3, 6, 32). A couple of studies by Wang et al. observed a selectivity of 405-nm blue light for *N. gonorrhoeae* cells over human vaginal epithelial cells, which supports its potential application as a treatment for *gonococcal* infections (3, 6). These studies include analysis of fluorescence emission spectra and ultra-performance liquid chromatography of normal human vaginal epithelial cells (VK2/E6E7) after exposure to 405 nm blue light (3). The co-cultures of *N. gonorrhoeae* cells and vaginal epithelial cells exposed to 108 J/cm^2 405 nm light showed a substantial increase in non-viable *N. gonorrhoeae* cells (from 32.85 $\pm$ 7.70% before light exposure to 78.15 $\pm$ 6.52% after light exposure), with no notable increase in the percentage of non-viable vaginal epithelial cells (3). Fluorescence emission spectra showed a small peak at 460 nm, indicating the presence of flavins, and otherwise minimal absorbance between 350 and 700-nm, indicating minimal presence of porphyrins (3).

The ultra-performance liquid chromatography analysis showed that VK2/E6E7 vaginal epithelial cells had substantially lower concentrations of endogenous flavins FAD, FMN, and riboflavin compared to ATCC 700825 *N. gonorrhoeae* cells with total flavin concentrations of 28.12 and 363.63 nmol/g respectively (3). Thus, it appears that the selective inactivation of *N. gonorrhoeae* cells over normal human vaginal epithelial cells upon exposure to 405 nm light can be explained by the increased presence of endogenous photosensitizers in the bacterial cells (3). Investigators proposed administration of 405 nm light via a laser with an optical fiber diffuser directed to the vaginal canal, similar to the present mode of administration of photodynamic therapy for treatment of condyloma acuminata caused by a human papilloma virus (HPV) in the vagina (3); further investigations by the same authors concluded that although infected vaginal epithelial cells were more vulnerable to 405 nm light than non-infected vaginal epithelial cells, the greater susceptibility of *N. gonorrhoeae* cells to 405 nm light indicated a possible therapeutic window of 405 nm light exposure whereby *N. gonorrhoeae* would be selectively inactivated, and the infected vaginal epithelial cells would be largely unharmed (6).

In a 2016 study investigating the potential of 415 nm light as a treatment for *C. albicans* keratitis, *C. albicans* was observed to be substantially more sensitive to photoinactivation than human keratinocytes, further reinforcing the idea of a therapeutic window in which the blue light can eliminate pathogens without harming normal human cells (2). Ferrer-Espada et al. and Liu et al. both observed that at an exposure dose of 216 J/cm^2 of the blue light, there was no significant reduction in the viability of human keratinocytes (32, 33), whereas the same dosage of light caused significant reduction of viability in the polymicrobial biofilms (33). A greater dosage of 405 nm light, 500 J/cm^2 was observed to cause cytotoxicity in the human keratinocyte cells (33). Another study using 415 nm blue light also observed multidrug-resistant *A. baumannii* to be substantially more susceptible to photoinactivation compared to human keratinocytes (17).

The blue light potential in prevention and treatment of eye infections

Recent studies have explored the potential application of the blue light for prevention and treatment of infection relating to the eyes (25, 43–45). Zhu et al. tested the efficacy of 415 nm light for the treatment of keratitis in an *ex vivo* rabbit model and an *in vivo* mouse model, where the causative agent was a bioluminescent strain of *P. aeruginosa* (43). In the experiment with *ex vivo* rabbit corneas, it was seen that the blue light treatment effected the greatest extent of reduction of bacterial luminescence when administered 6 h after inoculation, compared to 24 h after inoculation (43). There was no recurrence of infection observed in the rabbit corneas after antimicrobial light treatment (43). A similar *ex vivo* study of keratitis induced in rabbit corneas with a bioluminescent strain of *Candida albicans* found that 405 nm light was effective in substantially reducing the fungal infection (25). In the *in vivo* mouse study, complete bacterial luminescence eradication was achieved with treatment both 6 and 24 h after inoculation, although recurrence of infection was observed in both groups with the 24-h post inoculation treatment group exhibiting the more severe recurrence of infection (43). The observation that infection recurred in the *in vivo* experiment with mice and not in the *ex vivo* experiment with rabbit corneas suggests that the eyelids of mice in the *in vivo* experiments may have covered parts of the cornea during the 415 nm light exposure, thus preventing all the bacteria from being exposed to the light (43). The findings from this study provide evidence of the potential of 415 nm light as a treatment for *P. aeruginosa* keratitis, although further studies are needed to establish sound methods of adequately irradiating all the cornea in the *in vivo* context and mitigating the recurrence of infection after light treatment.

Discussion

This review has presented an overview of the existing understanding of visible blue light about its mechanism of microbicidal action, efficacy with inactivating different pathogens and degrading contaminants, as a potential multipurpose microbicidal tool for applications in clinical medicine and public health contexts, while providing safety for host cells or human exposure, unlike ultraviolet light (UV)-based technologies that also damage host environment. This study has also provided an assessment of the viability of potentially implementing the blue light as a disinfecting protocol in settings such as environmental decontamination, food safety, and treatment of localized infections.

Bacteria are the most thoroughly investigated and well-understood pathogen as a target of the blue light for bactericidal purposes. A widely accepted theory of the mechanism of inactivation of bacteria is that the blue light causes the photoexcitation of endogenous photosensitizers, namely porphyrins and flavins, which then precipitate the release of reactive oxygen species leading to toxicity and cell death (1, 3). There also appears to be consensus in the field that the sensitivity of a bacterium to a given wavelength of visible blue light may be affected by the type and prevalence of endogenous photosensitizers within the bacterium, and the wavelength of peak absorbance specific to the endogenous photosensitizers that are present (1, 3). Porphyrins have been noted to have peak absorbance around a wavelength of 405-nm, and flavins around 470 nm, and the relative abundance of these two endogenous photosensitizers may contribute to

the sensitivity of the bacterium to photoinactivation by certain wavelengths of visible blue light (1, 3). While porphyrins and flavins seem to be the most ubiquitous photosensitizers, there has also been evidence of the possible involvement of other photosensitizers in the photoinactivation of bacteria by antimicrobial blue light (1). An observed characteristic of bacterial inactivation by 405 nm antimicrobial blue light across studies is the greater susceptibility of gram-positive bacteria compared to gram-negative bacteria, with gram-negative bacteria generally requiring greater doses of light for inactivation (24). Antimicrobial blue light has also shown promise for addressing the issue of bacterial resistance, as studies using *N. gonorrhoeae* and *S. aureus* have shown no significant change in bacterial reduction in response to 405 nm light even after repetitive exposure to successive cycles of high-intensity light (5, 6). The finding that *S. aureus* exposed to 405 nm light at a low-level during culture exhibited a level of tolerance to inactivation by high-intensity 405 nm light suggests that bactericidal levels of light should be used in practice and indicates a need for further investigation of the mechanism through which this tolerance is developed (5).

Studies with fungi have shown antimicrobial blue light to have significant efficacy with reducing fungal burden (2, 25, 26). The presence of endogenous photosensitizers including porphyrins and flavins in *C. albicans* has been observed with fluorescence spectroscopy, which suggests the possibility that these photosensitizers play a role in the photoinactivation of *C. albicans*, as has been theorized to be the mechanism of photoinactivation in bacteria (2). Studies using antimicrobial blue light of wavelength 405 and 415 nm have observed *C. albicans*' susceptibility at both wavelengths (2, 25). There is a need for further investigation of fungal inactivation by antimicrobial blue light to characterize the effects of melanization by fungi in the context of infection, as this could potentially limit the efficacy of antimicrobial blue light (2). While there is evidence of fungal susceptibility to antimicrobial blue light, further investigation will be necessary to determine whether antimicrobial blue light is a viable antifungal treatment in practice.

Studies of viral inactivation by antimicrobial blue light also show some degree of antiviral efficacy. Because virions, unlike bacteria and fungi, do not contain endogenous porphyrins, there is relatively more ambiguity surrounding the mechanism and efficacy of photoinactivation (28). It has been observed that photoinactivation of viruses by antimicrobial blue light in the absence of other factors is limited but is enhanced in the presence of singlet oxygen enhancers or nutrient-rich media containing photosensitive compounds (27, 28).

The blue light has also been investigated as a tool for eliminating biofilms, which are of significant concern in contexts including environmental decontamination and treatment of localized infections due to their characteristic durability and decreased sensitivity to conventional antimicrobials (29, 30, 32). Studies show that treatment with visible blue light results in significant reduction in bacterial CFU when applied to monomicrobial and polymicrobial biofilms (32, 33, 35).

Several studies have also shown that visible blue light could be a promising tool for environmental decontamination and non-clinical applications. Studies in food safety have shown visible blue light to be effective in reducing the bacterial or viral burden on the surface of food products, although there is a need for more research to optimize the antimicrobial effects and understand potential effects on the quality of the food products themselves (24, 28). The blue light has been shown in multiple studies to be effective for reducing the bacterial burden within the clinical environment, as seen in studies of hospital isolation rooms, inpatient and outpatient burn treatment settings, and the hospital intensive care unit (12, 22, 37, 39). There appears to be agreement amongst studies that visible blue light is a viable tool that could potentially supplement existing hospital decontamination practices and help reduce the spread of nosocomial infections (12, 22, 37, 39). In addition, visible blue light has been shown to be effective when used in conjunction with a novel photocatalyst to degrade gaseous formaldehyde, a major contributor to indoor air pollution that is injurious to health (42).

Numerous studies have assessed the potential of the blue light for use in clinical applications, including treatment of gonococcal infections, localized infections, and wound healing. A common finding among these studies is that pathogens appear to have greater susceptibility to visible blue light than normal host cells, such that there is a therapeutic window of exposure within which the pathogen would be selectively inactivated, and the host cells preserved (2, 3, 6). These findings suggest that the blue light could indeed be a viable treatment for infection in the clinical setting. The observation of recurrence of infection after light treatment seen in more than one *in vivo* study is of concern and suggests that further investigation is needed to quantitate the prevalence of pathogen more effectively in the target area and more effectively administer the light to the entirety of the infected area (2, 43).

The existing literature supports the efficacy of visible blue light as an microbicidal tool in several applications including environmental decontamination and treatment of clinically relevant pathogens. It has been widely observed that although human cells are vulnerable to visible blue light at high doses, the doses that are lethal to many clinically relevant pathogens do not appear to cause damage to human cells (2, 3, 6). Although further studies will be needed to determine with more certainty the impact on normal human tissue and disease progression, current evidence points toward the viability of visible blue light for treatment of infections including otitis media, keratitis, gonococcal infections, and wound infections. Multiple studies have observed the blue light to have efficacy in inactivating multidrug-resistant bacteria, suggesting that the use of this light can potentially help to slow the spread of antibiotic resistance and untreatable infections (18, 20, 21, 23).

Further research is necessary to adequately understand the mechanism of inactivation for fungal and viral pathogens to better inform the way the blue light can be optimized to eliminate these pathogens in contexts such as the localized treatment of infections and decontamination of food and food processing environments. Regarding the application of the blue light to disinfection of produce and other food products, further investigation will be needed to determine any adverse impacts on the nutritional content and shelf life of the light-treated foods. Another direction for future research in the use of the blue light as an microbicidal tool is to further identify new factors that can be used in conjunction with the blue light to potentiate and enhance its microbicidal effects.

Author contributions

DH received guidance and mentoring by CA in writing the manuscript. CA reviewed and edited the manuscript for publication. Both authors approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Incidence of postpartum infections and outcomes associated with antibiotic prophylaxis after normal vaginal birth

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Antibiotic consumption accounted for approximately 15–20% of total drug costs in Thailand. From 2017 to 2018, 24.86% of Thai women who experienced vaginal delivery during normal term labour received antibiotics for postpartum infection. The Thai national practice guidelines set the target use of antibiotic prophylaxis in women following vaginal delivery of normal term labour to be no more than 10%. This study aimed to determine the incidence of postpartum infections and the outcomes and factors associated with antibiotic prophylaxis in women following vaginal delivery. The prospective cohort study was collected from 909 eligible patients who delivered infants in 7 secondary hospitals in Chiang Mai from July 2020 to February 2021. Antibiotic prescribing data and infections in women experiencing vaginal delivery during normal term labour were collected. The incidence of postpartum infections was calculated at 2 periods, 48 h and 6 weeks, after labour. Factors associated with the prescription of antibiotic prophylaxis in vaginal delivery were analysed using multivariate logistic regression. The results showed that the prevalence of antibiotic prescribing was 12.87% in a cohort of 117 patients. Postpartum infection was reported in 3 of 117 patients with antibiotics prophylaxis and 11 of 792 without antibiotics, with no statistically significant difference (RR: 1.04, 95% CI: 0.26–4.14; p = 0.956). Postpartum hygiene self-care practices were collected in the 6th week. The results found that there were no statistical differences in mean scores for all questions on postpartum hygiene self-care practices between the infected and non-infected groups (p-value $>$ 0.05). One of the factors associated with antibiotic prophylaxis was third to fourth degree of tear and episiotomy (OR: 7.72, 95% CI: 1.13–52.75; p = 0.037 and OR: 2.41, 95% CI: 1.24–4.70; p = 0.010, respectively). There was no significance difference in postpartum infection among patients receiving antibiotic and those who did not receive antibiotics. Third to fourth degree of

tear and episiotomy were significantly factors related to antibiotic prophylaxis in women with vaginal delivery after labour. This study supports practice guidelines and helps healthcare team to be assured on the use of antibiotics in no more than 10% of women experiencing normal vaginal delivery.

KEYWORDS

antibiotic prescription, antibiotic prophylaxis, women with vaginal delivery, rational drug use, postpartum infections

Introduction

The World Health Organisation (WHO) reported that more than half of patients receive antibiotics inappropriately (1), particularly in common conditions such as common cold, acute diarrhoea, fresh wounds, and normal vaginal delivery. Particularly, antibiotics had been prescribed without indication leading to overuse of antibiotics to patients unnecessarily, and patients sometimes stop taking the antibiotics before the end of the treatment. These affect the public health system, causing an increase in antimicrobial resistance (AMR) situations (2).

Antibiotic consumption accounted for approximately 15–20% of total drug costs in Thailand (3). The Ministry of Public Health implemented strategic policy actions to alleviate, prevent and control AMR (4). A rational drug use (RDU) programme was introduced in 2013, by the FDA sub-committee promoting responsible drug use, to promote multi-sectoral collaboration to reduce antimicrobial consumption and improve public awareness; this started with antibiotic usage in upper respiratory tract infections, acute diarrhoea and fresh traumatic wounds and antibiotic prophylaxis in vaginal delivery in normal term labour (5).

From 2017 to 2018, 24.86% of Thai women experiencing vaginal delivery during normal term labour received antibiotics for postpartum infection (6). Postpartum infection is one of the leading causes of death in women after giving birth. The overall incidence of postpartum sepsis was 6%, with the incidence of sepsis following caesarean section and vaginal delivery being 7.4 and 5.5%, respectively (7). Factors associated with postpartum infection include obesity, BMI > 30, anaemia, diabetes, smoking, pregnancy over 35 years, employment, the degree of perineal wound, delivery time (more than 12 h), frequency of vaginal examination (more than 5 times) and use of equipment for delivery (8–10).

Prescribing antibiotics reduced the mortality rate and decreased the incidence of postpartum infection, in particular, surgical and vaginal deliveries with grade 3–4 perineal wounds. The study found that antibiotics in women with grade 3–4 perineal wounds were able to reduce the incidence of wound infections (RR: 0.34, 95% CI: 0.12–0.96) (11). The report from a tertiary care hospital in Thailand found that 22.7% of women received antibiotics for postpartum prophylaxis and most of antibiotics were amoxicillin (12).

The RDU programme is focused as a health service plan in Thailand (4). Practice guidelines (5) set the use of antibiotic prophylaxis in women following vaginal delivery during normal term labour to be no more than 10%. According to WHO recommendations (12) for the prevention and treatment of maternal peripartum infections, routine antibiotic prophylaxis is not recommended for women with uncomplicated vaginal births. The prevalence of antibiotic prescribing in Thailand for prophylaxis during vaginal delivery was higher than that in the RDU practice guidelines; also, there have been a limited number of studies identifying factors associated with antibiotic prescribing and the incidence of postpartum infection between antibiotics prophylaxis versus no prophylaxis in women following vaginal delivery. The aim of this study was to determine the incidence of postpartum infections and outcomes and factors associated with antibiotic prophylaxis in women following normal vaginal delivery.

Materials and methods

Study design and setting

This was a multi-centre prospective cohort study among women admitted for normal vaginal delivery at seven secondary care hospitals in Chiang Mai, Thailand. All women attending the study hospitals for vaginal delivery of normal term labour from July 2020 to February 2021 were recruited into the study. Women with a normal vaginal delivery, no pyrexia (body temperature < 38°C when measured in the armpits or < 38.5°C when measured in the oral cavity) and amniotic sac ruptures no later than 24 h before delivery were included in the study. Women were excluded from the study if vacuum extraction (forceps delivery) was used for delivery, antepartum or postpartum haemorrhage, and diagnosis of Group B *streptococcus* infection or the microbial cultured presence of Group B *streptococcus*. Sample size was calculated using 2-sample non-inferiority or superiority of postpartum infections

during 6-week follow-up after delivery between antibiotic and non- antibiotic groups.

Exposure of antibiotic prescribing for postpartum infection prophylaxis

Maternal intrapartum antibiotic prophylaxis (IAP) was identified as a systemic or oral antibiotic was prescribed for women with normal vaginal delivery for an indication to prevent postpartum infections and documented in hospital medical records. Antibiotic prescribing for women after delivery at discharge was also identified as antibiotic prophylaxis. Women received antibiotic for postpartum infection prophylaxis was antibiotic group. Women with no record of antibiotic prescribing for postpartum infection prophylaxis was considered a non-antibiotic or control group.

Postpartum infections as study outcome

The criteria for determining postpartum infection were the presence of at least 2 clinical symptoms, i.e., abnormal vaginal discharge, pyrexia (oral temperature measurement more than 38.5°C), abnormal smell/foul odour discharge, delay in uterine involution (less than 2 cm per day during the first 8 days after delivery), and pelvic pain, assessed by the trained clinicians. The signs and symptoms of infection were reviewed by gynaecologists to confirmed postpartum infections at 48 h after normal delivery and at the 6-week follow-up visit.

Data collections

The study was approved by the research ethics committee, Faculty of Pharmacy, Chiang Mai University (No. 12/2563). Written informed consent was obtained from the participants at enrolment. Trained clinicians collected data from medical records, including demographic data, data related to delivery, antibiotic prescribing, and sign and symptoms of postpartum infections at 48-h and 6-week follow-up at outpatient clinic for puerperium care. Participating clinicians were trained for participant recruitment and data collection. Factors associated with antibiotic prophylaxis were collected, including occupation, age, duration of labour, anaemia, completed antenatal care, BMI, total number of pregnancies, degree of vaginal tear. A standardised self-administered questionnaire on self-care practices and hygiene was completed by women after hospital discharge using during home stay before the 6-week follow-up. Postpartum hygiene self-care practices during 6 weeks after delivery were assessed using a 5-item self-administered questionnaire on hygienic practices including showering, handwashing, perineal hygiene, changing sanitary pad, and delaying sexual relation. A 5-point Likert scale was used to rate the practices, ranging from 1 = "never," 2 = "rarely," 3 = "sometimes," 4 = "often," and 5 = "always." The reliability test revealed that the Cronbach's alpha coefficient of this questionnaire was 0.73. The responses were grouped into "often-always" and "never – sometimes."

Data analysis

Descriptive statistics were used to describe patient characteristics. To test the differences of characteristics of women received or not received antibiotic prophylaxis for postpartum infection, student's *t*-test was used for continuous variables and Chi-square test for categorical variables. Relative risk (RR) was calculated at 48 h and 6 weeks after delivery to indicate the incidence of postpartum infections in antibiotic and non-antibiotic groups. The study was powered to detect an 80% difference in the postpartum infection outcome. Multivariable logistic regression was performed to adjust for potential confounders, such as maternal age, history of self-administered antibiotic, length of hospital stay, duration of labour, degree of vaginal tear, anaemia, and number of pregnancies. Multivariable logistic regression was used to identify factors of antibiotic prescribing for postpartum infection prophylaxis. Statistical significance was determined by $p < 0.05$ for the study outcome. All analyses were conducted using STATA version 14.0 (STATA Corp., College Station, TX, United States).

Results

The prospective cohort study consisted of 909 women who had a normal vaginal delivery at the secondary care hospital, Chiang Mai province, Thailand, between July 2020 and February 2021. Statistical analysis, including univariate and multivariable logistic regression were conducted to assess the incidence of postpartum infection and factors associated with antibiotic prophylaxis (**Figure 1**).

Baseline characteristics

Throughout the study period, of the 909 cases, 117 women experiencing normal vaginal delivery were prescribed antibiotics for the prevention of postpartum infection, corresponding to 12.87%. Both groups were comparable in all baseline parameters, with no statistically significant difference. The majority of participants were aged between 20 and 34 years-old, with a mean age of 24.80 ± 6.47 years in the antibiotic

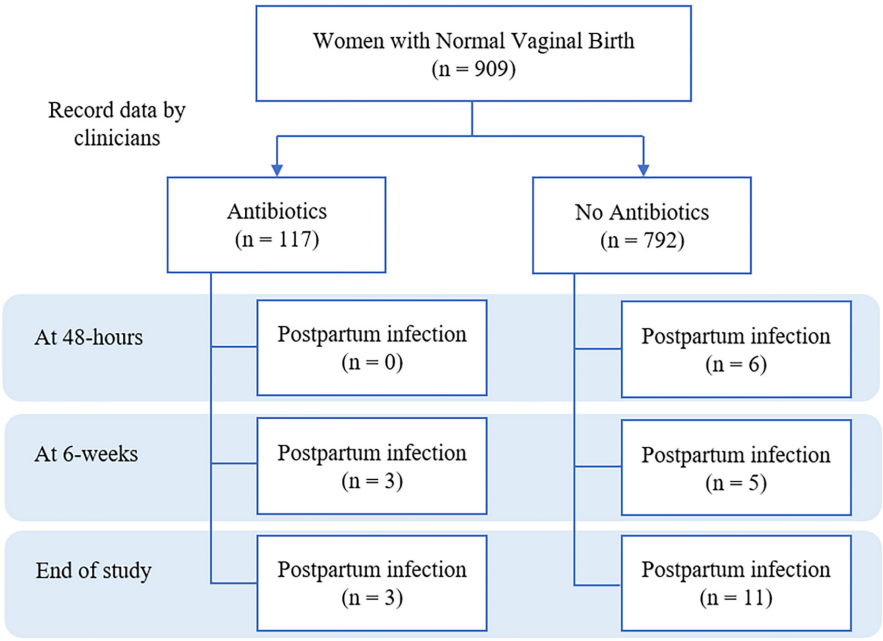

FIGURE 1
Flow chart of study on incidence of postpartum infection between antibiotics and no antibiotics prescribing.

group and 25.26 ± 6.75 years in non-antibiotic group. Most participants had a BMI ≤ 30 kg/m^2, were unemployed and completed antenatal care 5 times (Table 1). However, patients were excluded from the study due to the presence of frequent risk factors during pregnancy or delivery for infection in the postpartum period, such as postpartum haemorrhage, for which prophylactic antibiotics are recommended. Furthermore, none of the study participants had diabetes or a smoking history.

Incidence of postpartum infection

At the end of study, 14 of the 909 cases (1.54%) were diagnosed with postpartum infection. It was found that all of them had abnormal vaginal discharge, with symptoms presenting according to other diagnostic criteria. Most of the infected women had an abnormal smell/foul odour discharge and pelvic pain. By dividing at the time of inspected at 48 h and 6 weeks, there were 6 cases and 8 cases diagnosed postpartum infection at those time, respectively.

The results on the incidence of postpartum infection between the antibiotic and non-antibiotic groups were reported. In the antibiotic group, 3 of the 117 cases had postpartum infections, while there were 11 out of 792 cases in the non-prescribed antibiotic group who had postpartum infections (Table 2). The incidence rate of postpartum infections was 18.3 per person-month in the antibiotic group and 10.0 per person-month in the non-antibiotic group, respectively.

The most commonly prescribed antibiotic in the hospital for prophylactic infection in 117 cases was oral amoxicillin, with a usage level of 77 times in 63 cases, as a percentage 53.8% in the antibiotic group. The next highest were ampicillin and ceftriaxone in 31 cases (26.5%) and 14 cases (12%), respectively.

Factors associated with antibiotic prophylaxis

Univariable and multivariable logistic regression were used to determine factors associated with antibiotic prescription for prophylaxis postpartum infection in women following normal vaginal delivery. The results showed that the degree of tear as third to fourth degree and episiotomy were a statistically significant factor for antibiotic prescribing in women experiencing normal vaginal delivery (OR: 7.72, 95% CI: 1.13–52.75; $p = 0.037$ and OR: 2.41, 95% CI: 1.24–4.70; $p = 0.010$, respectively; Table 3).

Postpartum hygiene self-care practices among postpartum infection and non-infection groups

From a total of 909, there were 428 women responded to the questionnaire, 5 women in the infection group and 423 women in the non-infection group. The results indicated that there were

TABLE 1 Baseline characteristics.

Data	Antibiotics (n = 117)		No antibiotics (n = 792)		p-value
	No.	(%)	No.	(%)	
Hospital stay (day)					
(Mean ± SD)	2.46	±0.93	2.20	±0.63	0.008
Occupation					
Yes	21	(18.0)	164	(20.7)	0.489
No	96	(82.0)	628	(79.3)	
Age (year)					
≤19	24	(20.5)	164	(21.3)	0.898
20–34	81	(69.2)	535	(69.7)	
≥35	12	(10.3)	69	(9.0)	
Average (Mean ± SD)	24.80	±6.47	25.26	±6.75	0.498
Duration of labour					
<12 h	86	(74.8)	597	(78.9)	0.322
≥12 h	29	(25.2)	160	(21.1)	
Median (IQR)	6.3	(10.0)	6	(7.2)	0.719
Anaemia					
Hb < 11 g/dl	13	(11.7)	110	(15.7)	0.282
Hb ≥ 11 g/dl	98	(88.3)	593	(84.3)	
Antenatal care					
Complete (5 times)	63	(57.8)	494	(64.5)	0.174
Incomplete	46	(42.2)	272	(35.5)	
BMI (kg/m^2)					
≤30	110	(94.8)	694	(89.4)	0.069
>30	6	(5.2)	82	(10.6)	
Total number of pregnancies					
1 time	57	(49.6)	301	(38.5)	0.070
2–4 times	56	(48.7)	458	(58.6)	
≥5 times	2	(1.7)	23	(2.9)	
Degree of vaginal tear					
None	12	(10.2)	207	(26.1)	<0.001
First-second degree	25	(21.4)	211	(26.7)	
Third-fourth degree	2	(1.7)	4	(0.5)	
Episiotomy	78	(66.7)	370	(46.7)	

Hb: haemoglobin.

TABLE 2 Incidence rates of postpartum infections by antibiotic prophylaxis status.

Outcome parameter	No. of patients (%)		Unadjusted risk ratio (95% CI)	P value	Adjusted * risk ratio (95% CI)	P value
	Antibiotic (n = 117)	Non-antibiotic (n = 792)				
Postpartum infection	3 (2.6)	11 (1.4)	1.85 (0.52–6.52)	0.341	1.04 (0.26–4.14)	0.956
Postpartum infection by degree of vaginal tear						
None	0	0	NA		NA	
First-Second degree	2 (1.7)	1 (0.1)	0.52 (0.14–1.84)	0.308	0.67 (0.17–2.57)	0.563
Third-fourth degree	0	0	NA		NA	
Episiotomy	1 (0.9)	10 (1.3)	NA		NA	

*Adjusted for covariates: antibiotic prophylaxis, age, hospital stays, duration of labour, degree of vaginal tear, and total number of pregnancies.

TABLE 3 Factors associated with antibiotic prescribing for postpartum infection prophylaxis among women with normal vaginal delivery.

Factors	Unadjusted OR (95% CI)	*p*-value	Adjusted OR* (95% CI)	*p*-value
Occupation	0.84 (0.51–1.38)	0.490	0.86 (0.49–1.52)	0.616
Age ≥ 35 years	1.16 (0.61–2.21)	0.657	1.52 (0.71–3.26)	0.286
Duration of labour > 12 h	1.26 (0.80–1.98)	0.323	1.27 (0.76–2.12)	0.357
Anaemia (Hb < 11 g/dl)	1.40 (0.76–2.58)	0.284	1.43 (0.75–2.76)	0.280
Completed antenatal care (≥5 times)	0.75 (0.50–1.13)	0.175	0.66 (0.42–1.02)	0.064
BMI > 30 (kg/m^2)	0.46 (0.20–1.08)	0.076	0.52 (0.21–1.25)	0.145
Total number of pregnancies				
1 time	1.00		1.00	
2–4 times	0.64 (0.43–0.96)	0.031	0.66 (0.42–1.05)	0.080
≥5 times	0.46 (0.10–2.00)	0.300	0.66 (0.14–3.25)	0.614
Degree of vaginal tear				
None	1.00		1.00	
First-second degree	2.04 (1.00–4.18)	0.050	1.71 (0.80–3.670)	0.165
Third-fourth degree	8.62 (1.43–51.88)	0.019	7.72 (1.13–52.75)	0.037
Episiotomy	3.64 (1.93–6.84)	<0.001	2.41 (1.24–4.70)	0.010

Hb: haemoglobin. *Adjusted for covariates: antibiotic prophylaxis, age, hospital stays, duration of labour, degree of vaginal tear, and total number of pregnancies.

no statistical differences of the proportions of postpartum self-care practices and the mean scores of all items between the infected and non-infected groups (p-value > 0.05; Table 4).

Discussion

This study found that 117 of a total 909 women with normal vaginal delivery (12.87%) were prescribed antibiotics for postpartum infection prophylaxis, of which 53.8% of the women received oral amoxicillin. Postpartum infection was found in 3 of 117 women treated with antibiotics and 11 of 792 women with no antibiotic prophylaxis (OR: 1.04, 95% CI: 0.26–4.14; p = 0.956). At the six-week follow-up visit, the mean scores of postpartum hygiene and self-care were not different between women received antibiotics and no antibiotics (p-value > 0.05). The level of tear in third to fourth degree (OR: 7.72, 95% CI: 1.13–52.75; p = 0.037) and episiotomy (OR: 2.41, 95% CI: 1.24–4.70; p = 0.010) were the factors associated with antibiotic prescribing for postpartum infection prophylaxis.

An Indian study (13) found that antibiotics were prescribed in as many as 87% of women. The majority of antibiotics prescribed (35%), were third generation cephalosporins. The study at a tertiary care hospital in Thailand (12) reported that 22% of patients were prescribed antibiotics for prevent postpartum infections, and the most commonly used antibiotic was oral amoxicillin (93.62% of cases). It is found that the antibiotic prescribing rate in India is much higher than Thailand. This might be due to the public health problem in the country, with a high rate of maternal infection and which leads to mortality in India. Thus, antibiotic treatment aims to reduce the rate of infection. However, antibiotic prescription in Thailand is still higher than recommendations from the Thai RDU hospital programme which recommended antibiotic prescription of no more than 10% in women with normal vaginal delivery. Considering the prescription of antibiotics in Thailand, oral amoxicillin is not suitable for use in the prevention of postpartum infections because it is ineffective on the most common organisms in postpartum infection, including *Staphylococcus aureus*, *Escherichia coli*, group B *streptococci* and anaerobes, such as *Bacteroides* spp. (14, 15). The rational drug use guidelines of Thailand (4) and WHO guidelines (1) recommend the use of single intravenous antibiotics, 1–2 g of cefazolin or 3 g of ampicillin-sulbactam, within 60 min prior to suturing in cases of third to fourth degree tears. In the case of hypersensitivity to penicillin, 600–900 mg of clindamycin is recommended. This is similar to the recommendations of the American College of Obstetricians and Gynaecologists (16) which recommends the use of first-generation cephalosporin or clindamycin for penicillin allergy. As a result, only 1.7% of antibiotic prescriptions use a single injection of cefazolin and this seemed to be an inappropriate use of antibiotic prescriptions for postpartum prophylaxis in Thailand.

The incidence of postpartum infections in women with normal vaginal delivery was followed-up at 48 h and 6 weeks after delivery by trained nurses. It was found that 1.54%, or 14 cases out of the 909 cases, were diagnosed with postpartum infections in this study. The study by Yokoe et al. (7) from the United States reported overall rates of infection after childbirth and vaginal delivery of 6 and 5.5%, respectively. This might be the postpartum infection diagnosis and group designation which included other in-hospital diagnoses, including emergency rooms and outpatient departments, such as mastitis, urinary tract infections, urinary

TABLE 4 Postpartum hygiene self-care practices in infected and non-infected groups.

Item	Postpartum hygiene self-care practices	Infection (n = 5)		Non-infection (n = 423)		p-value
		No.	(%)	No.	(%)	
1	**Take a shower at least twice a day**					
	Often – Always	1	(20.0)	219	(51.8)	0.204*
	Never – Sometimes	4	(80.0)	204	(48.2)	
	Average score (Mean ± SD)	3.20	±1.10	3.47	±0.97	0.543**
2	**Wash hands with soap and water regularly or often use hand sanitizer after touching objects and surfaces, using the toilet, or changing diaper.**					
	Often – Always	3	(60.0)	283	(66.9)	0.668*
	Never – Sometimes	2	(40.0)	140	(33.1)	
	Average score (Mean ± SD)	4.20	±1.10	3.85	±0.84	0.352**
3	**Cleaning perineum after urination by wiping from front to back**					
	Often – Always	3	(60.0)	305	(72.1)	0.623*
	Never – Sometimes	2	(40.0)	118	(27.9)	
	Average score (Mean ± SD)	4.20	±1.10	3.95	±0.78	0.480**
‘4	**Changing sanitary pad when soiled or every 3–4 h by pulling the pad from front to back**					
	Often – Always	3	(60.0)	334	(79.0)	0.288*
	Never – Sometimes	2	(40.0)	89	(21.0)	
	Average score (Mean ± SD)	4.00	±1.00	4.14	±0.78	0.682**
5	**Avoiding sexual relations for 4–6 weeks after childbirth**					
	Often – Always	5	(100.0)	387	(91.5)	N/A
	Never – Sometimes	0	(0.0)	36	(8.5)	
	Average score (Median, IQR)	5.00	0	5.00	1	0.131**
	Total	20.6	±3.50	20.0	±2.93	0.643

*Chi-squared test. **Student’s t-test.

tract infections, surgical site infection, endometritis and perineal wound infection. More than 80% of them had mastitis and urinary tract infections. However, the study did not reveal the number of pregnant women who received antibiotics and types of antibiotics used to prevent infections. Another study in tertiary care hospitals Thailand at Mahasarakham Hospital (17) used retrospective data between October 1 2015 and April 30 2018 and found that 2 out of 3,660 women reported postpartum infection after normal vaginal delivery, representing a rate of 0.05%. Since infected individuals might go to another hospital, preventing data collection from medical records when patients returned for follow-up after delivery, the infection rate was lower than usual. Moreover, Mahasarakham Hospital is a tertiary care hospital with a difference from this research study, with eight secondary care hospitals, as there was an obstetrician and gynaecologist who performed the delivery in this population of pregnant women. When considering the antibiotics prescribed, the tertiary care study prescribed oral amoxicillin in about 51.1% of cases, similar to this multi-research site study (17).

When analysing data of postpartum infections, it was shown that 3 of the 117 cases (or 2.6%) in the antibiotic-treated group were infected compared to the non-antibiotic groups, with 11 out of 792 cases (1.4%). There was no statistically significant difference in the proportion of postpartum infections between the two groups (p = 0.956). The systematic review by Bonet (23) on the use of antibiotics to prevent infection in women after delivery found that the risk of urinary tract infections, wound infections and length of hospital stay in women treated with antibiotics were not different when compared to those who received placebo or non-antibiotics. In the study by Tandon and Dalal (18), the symptoms of infection were 0.7 and 2% in the antibiotic-treated group and the untreated group, respectively. There was no statistically significant difference in symptoms of infection in either group (p < 0.622). Another study in Thailand (19) compared complication outcomes in the antibiotic-treated group versus the no antibiotic group and found that neither group had complications in postpartum sepsis. Postpartum infections, between 57 women treated with amoxicillin compared to 56 women without amoxicillin, were not found in either group (20). The infection assessment criteria were similar in this study. The results of these studies showed that the outcome of postpartum infection was not different between antibiotic and non-antibiotic use in women with normal delivery. Therefore, antibiotic prophylaxis should not be

given to prevent postpartum infections in women who have a normal vaginal delivery, except in patients with third to fourth degree tears. The WHO (1) and Thailand's Rational prescribing guidelines (5) recommended a single intravenous injection antibiotic within 60 min before suturing with lists of antibiotics, including cefazolin 1–2 g or ampicillin-sulbactam 3 g and clindamycin 600–900 mg, for patients who show hypersensitivity to penicillin. This was ineffective against the causative organisms of postpartum infections, including *Staphylococcus aureus*, *Escherichia coli* and *Streptococcus* species and anaerobic bacteria such as *Bacteroides* spp. Based on the findings of this study, two of the six cases of third to fourth degree tears were treated with oral amoxycillin, which abused the guidelines for antibiotic use in the prevention of postpartum infections. Additionally, there are no culture-confirmed bacterial pathogens causing infections in diagnosed cases with postpartum infections, which is a study limitation because these infections are likely under-reported in inpatient charts.

A study of factors associated with antibiotic prescription to prevent infection in women after normal vaginal delivery found that the factors associated with antibiotic prescription by doctors included the degree of tear, especially the level third to fourth degree of tear and episiotomy (p-value = 0.037 and p-value = 0.010). According to the study by Sharma et al. (21), 81.1% of women with perineal episiotomy were prescribed antibiotics. The most commonly prescribed antibiotics in the 5-day oral form were ampicillin, amoxicillin and cephalexin. The study by Bonet (24) reported that the use of antibiotics in episiotomy was not statistically different in infected wounds between the antibiotic-treated or non-antibiotic groups. WHO guidelines (1) do not recommend the prescription of antibiotics for the empirical treatment of postpartum infection in women with episiotomy lesions. Also, the American College of Obstetricians and Gynaecologists (22) does not recommend the use of antibiotics in the prevention of postpartum infection.

Conclusion

At the end of the study, 14 out of 909 cases (1.54%) were diagnosed with postpartum infection. The incidence of postpartum infection was 1.04 times (3/114 women) higher in the antibiotic group compared to the non-antibiotic group (11/781 women). There was no significance difference in the use of antibiotics or not for prophylaxis in postpartum infection. The degree of tear at the level of third to fourth degree and episiotomy are the factors considered when prescribing antibiotics to prevent infection in women following vaginal delivery. Evidence-based practice using data to empower and encourage healthcare teams in the awareness of prescribing antibiotics to no more than 10% of patients following vaginal delivery in normal term labour for the prevention of postpartum infection.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by ethics committee on human research of the Faculty of Pharmacy, Chiang Mai University (12/2563). The patients/participants provided their written informed consent to participate in this study.

Author contributions

TS, PK, and WK: conceptualization, methodology, writing – original draft, and writing – review and editing. TS: data curation, formal analysis, investigation, project administration, and software. PK and WK: supervision. SN: validation. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Assessment of antibiotic storage practices, knowledge, and awareness related to antibiotic uses and antibiotic resistance among household members in post-conflict areas of Pakistan: Bi-central study

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Background: The storage of antimicrobials at home is frequently in-practice in various developing countries, resulting an irrational use, antibiotic resistance, and toxicities. This condition may worsen more in conflict zones where health facilities are limited. This study aimed to determine the storage and use of leftover antibiotics among households (HHs) along with knowledge and awareness about antibiotics and antibiotic resistance (ABR).

Methods: A descriptive cross-sectional study design was employed. Members of HHs were invited to participate in the survey while using a convenient sampling technique. The data were obtained using a validated questionnaire and analyzed through SPSS.

Results: A total of 96 HHs were randomly selected from two districts ($n = 50$, $n = 46$), with most of the participants being men between the ages of 18 and 28 ($n = 45$, 46.9%) years. The majority of HHs ($n = 32$, 33.3%) had six to eight total family members, with one to two chronic diseases ($n = 63$, 65.6%), individual families ($n = 60$, 62.5%), and with ($n = 35$, 36.5%) LRTIs (lower respiratory tract infections). The HHs were aware of the word "antibiotic" ($n = 59$, 61.5%) and gave correct replies to amoxicillin as an antibiotic ($n = 42$, 43.8%); on the other hand, HHs also thought of paracetamol as an antibiotic ($n = 45$, 47.9%). They identified the most common brands of antibiotics easily, and a majority of them ($n = 69$, 71.9%) had never heard of ABR before and had lower levels of awareness. The most stored antibiotic at home (n=38, 39.6%) was azithromycin

(J01FA10). In addition, they had multiple needless (1–2, $n = 62$, 64.6%; 3–4, $n = 29$, 30.2%) and antibiotics in their houses. Age had a strong association ($p = 0.017$, H = 12) affected the mean knowledge scores related to antibiotic use. Association of education levels ($p = 0.001$, H = 52.8) and occupation ($p = 0.04$, H = 10) with proper antibiotics use were found to be significant. However, family members with more than one chronic illness ($p = 0.09$, H = 0.8) showed a significant relationship with their awareness of antibiotics.

Conclusion: Participants generally stored various antibiotics of different classes in their homes. Lack of knowledge related to the appropriate usage of antibiotics, use of leftover antibiotics, and awareness related to ABR were unknown to the participants.

KEYWORDS

antibiotics storage, antibiotic resistance, households, post-conflict areas, Pakistan

Introduction

A majority of households (HHs) around the world keep medicines on hand for a variety of reasons, including the treatment of both short-term and long-term illnesses, as well as for emergencies. If the directions for storage are not followed, drug stability may be impacted, which could result in ineffective therapy (1, 2). The therapeutic effectiveness of antimicrobial medicines is dependent on their capacity to selectively target invasive bacteria without damaging host cells, yet their manufacturing and stability are key challenges (3). However, the risk of accidental poisoning by unintentional users such as children has also been associated with drug sharing and reusing (2). Conversely, antibiotics can also have negative effects. The most frequent negative side effects related to antibiotic use include allergic responses, ototoxicity, hepatotoxicity, phototoxicity, and nephrotoxicity (4). Regardless, stockpiling of medicines in HHs has been on the rise, given the impact of violence and conflict in many parts of society, including the health sector. In war zones, antibiotic storage in HHs is particularly prevalent (5).

Conflict or war is described as "a condition of open, typically long-term combat between two parties or opponents." Conflicts have significantly harmed health systems across the world, resulting in delayed access to care, overcrowding, and shortages of medical supplies (6). As a result, the storage of antibiotics at home has grown with an aim to utilize any leftover antibiotics in the future. Unsupervised antibiotic storage leads to antibiotic abuse, which is a result of unneeded antibiotic storage in HHs in conflict zones worldwide (7, 8). In combat zones, self-medication with antibiotics is one type of abuse, which refers to treating self-diagnosed diseases without contacting a physician or under the supervision of a doctor and pharmacist (8, 9). Antibiotics are often purchased over-the-counter across the developed and developing world, with around half of all antibiotics received without a prescription (10, 11). According to microbiological data analysis by Médecins Sans Frontières (MSF), third-generation cephalosporin and carbapenem resistance is 86.2% and 4.3% respectively among Enterobacteriaceae isolates; MRSA (methicillin-resistant *Staphylococcus aureus*) is found in 60.5% patients, and resistance types and rates are similar in patients from Yemen, Syria, and Iraq (12). The National Institute of Health in Islamabad reported that conflict is believed to have led to widespread drug-resistant typhoid fever, which could affect over 2,000 individuals in Pakistan within 6 months. Only one oral antibiotic, azithromycin, was shown to be sensitive (13). Intravenous medications, for example, are costly and impractical in low-income nations. Antimicrobial self-medication has been linked to leftover antibiotics (14, 15). This means that keeping antibiotics in the home increases the likelihood of irrational antibiotic usage. Antibiotics are among the most routinely kept drugs in homes (16, 17). Studies conducted in the United Kingdom and Australia have shown that 19–47% of HHs have stored antibiotics at home including remnant, standby, and current antibiotics (18, 19). Antimicrobial/ABR is a significant public health issue that affects both developed and developing countries. Moreover, the incidence of ABR has risen in recent years, and this, along with the scarcity of antibiotics in the pipeline, has resulted in an ABR crisis. Available evidence indicates that 62% of antibiotics purchased in community pharmacies do not have a prescription (20). Furthermore, self-medication with antibiotics is common, ranging from 19 to 82% (14, 15, 21), and this is usually associated with inappropriate antibiotic use (15, 21). Globally, resistance patterns have been seen among the members of Enterobacteriaceae, *Mycobacterium tuberculosis, Staphylococcus aureus*, *Streptococcus pneumoniae,* and *Pseudomonas aeruginosa*. Various monitoring systems have been established to check for variations in how susceptible bacteria are to antimicrobial medications. Estimating the

worldwide incidence of AMR/ABR will be greatly aided by the availability of updated epidemiological data on frequently encountered diseases exhibiting resistance to antimicrobial agents (22). ABR, therefore, has emerged as one of the main issues facing developed and developing countries that require immediate action to stop the misuse of antibiotics.

Swat, a district in the Khyber Pakhtunkhwa region of Pakistan, has witnessed extensive armed conflict since 2007 (23). During the tumultuous conflict between militants and the army in Pakistan in 2009, more than two million people from the valley became internally displaced persons (IDPs) (24). These IDPs were asked to return in July 2009 after the government proclaimed the region peaceful, while the area's ongoing military presence and sporadic clashes between security forces and militants continue to cause societal unrest (25). Currently, the situation in the Swat valley is peaceful, and in the past decade, several infrastructural projects have been initiated in the health sector. However, more work is still needed, especially in rural areas. The objective of this study is to evaluate HH storage practices, uses, knowledge, and awareness about antibiotics and ABR in the post-conflict area of Swat and Shangla in Pakistan.

Methods

Study design and location

From November 2020 to April 2021, a bi-center cross-sectional interview-based survey was conducted in the rural parts of two post-conflict districts namely Swat and Shangla in Pakistan. Swat, formally known as the "Yousufzai State of Swat" (1849–1969), is a rural district in Malakand Division Khyber Pakhtunkhwa, Pakistan, with a population of 2.3 million people. About 86% of the total population lives in rural areas. District Shangla was once a part of Swat state but was carved out in 1995 as a separate district and adjunct to the Swat district having a population of 0.7 million (26). The current study is part of the main trial which contributes baseline data to the registered protocol (ChiCTR2000040453) and is a scheduled phase of the research project. The results that have been presented are following the EATSA protocol and serve as a foundation for the beginning of the main trial (27).

Study population

This study employed a door-to-door survey method, which is an excellent strategy for gathering meaningful measures of health. In the vicinity of both district rural regions, the HHs were prioritized for data collection. Permanent residents of the area or residents who have lived in the area for the past 5 years and do not have a disability were eligible to answer questions and respond to the research teams. Participants under the age of 18, as well as HHs with no male members and those who refused to consent, were all excluded from the study. Following their visits to each HH, the data collection teams (DCTs) received preliminary training to ensure that the norms and local traditions are considered. For a total of 2 weeks, DCTs received prior training daily throughout their working days.

Validity and reliability

The face and content validity of the questionnaires were evaluated with the help of specialists in the field. The study tool was piloted with a group of 15 HHs to determine its internal consistency. Visits to both research sites were part of the pilot project, which were utilized to validate the questionnaire. This part of the study provided feedback to the research team, confirming that they had a good understanding of the questions (Supplementary File 2).

The internal consistency of the amended questionnaire was calculated using Cronbach alpha on 22 HHs. The Cronbach alpha result (overall = 0.7; each, 0.69) was adequate and acceptable, indicating that the questionnaire was internally consistent.

Study instruments

Medical records were utilized as data sources, and all the information was self-reported by the participants. The questionnaire requested details on demographic factors (gender, age, location, occupation, education level, family status, and average monthly income), as well as respondents' awareness of antibiotics, ABR, and antibiotic storage procedures at their homes. Each question prompted replies in a variety of ways, including yes/no/don't know, closed-ended questions with numerous answers, and open alternatives. The questionnaire was developed using relevant information from prior studies on the subject (19, 28–32).

The final questionnaire had three sections. Section I sought demographic details and had a total of 10 questions. Section II had questions related to knowledge (antibiotics identification=4, antibiotics use = 6, and antibiotic resistance = 6) of antibiotics and 16 statements to which the respondents were asked to respond. Two false and two true (Amoxicillin is an antibiotic/Paracetamol is an antibiotic) statements were also included in the identification of antibiotics. Three false questions were asked about the use of antibiotics (antibiotics are often needed for cold and flu), and six questions were related to ABR (misuse of antibiotics can lead to ABR) with the correct approach. A total of eight statements were also employed for the assessment of overall HHs' knowledge of antibiotics use, ABR, and infectious diseases. The responses to these statements were evaluated (Likert scale). Section III

included 15 statements/questions related to the HH storage of antibiotics and factors related to storage conditions.

Translation

The questionnaire was translated by two native translators through the forward and backward translation methods. All data was gathered using a structured paper questionnaire with questions written in the local and national languages (Pashto/Urdu). Forward and reverse translation procedures were undertaken by experts due to the questionnaire's cultural adaption.

Sampling technique and sample size

A convenient sampling technique was adopted to collect data. We intended to recruit as many HHs as feasible over the course of the study to increase the sample's prospective representativeness. As a logical consequence, we did not determine the minimum sample size in advance (33). As a result, ninety-six HHs (50 in Swat and 46 in Shangla) was determined as the quota needs to represent a mix of geography, pharmacy type (independent and chain community pharmacies), and family size in the HH (large, medium, and small; combined or/ individual).

Data collection

A significant amount of group orientation was provided to the DCTs, covering areas like guidelines for conducting the interview and HHs survey, advice on proper attire to respect the locale's cultural norms, and other specifications. The DCTs were coached by researchers who were familiar with the clinical situations and the rural community environment. Before administering the survey, the researchers and DCTs visited 22 residences (Swat = 12 and Shangla = 10) in both districts jointly to create familiarity and confidence.

Quality control procedures and data management

Several efforts were made to achieve maximum standardization and preserve consistency in the data gathering. All data were assigned a special code of identification. The research team members entered the data and cross-checked it. All files were stored, and the confidentiality of the data was assured.

Statistical analysis

Statistical Package for the Social Sciences (SPSS version 21) was used for data entry and data compilation. Additional test statistics were employed to compare categorical variables, and descriptive statistical analyses were provided as frequencies (percentages). If $p>0.05$, differences were judged statistically significant. Due to the non-normal data distribution, continuous data were presented as median and interquartile ranges (IQRs). The comparison of continuous data across various samples was conducted through Kruskal–Wallis, and Mann–Whitney tests, where appropriate. Antibiotic use, identification, and ABR median scores (which measure associated characteristics) were also calculated and compared with demographic data such as age, gender, occupation, education level, the total number of families, average income, and family setup. The median scores and testing statistics scores for the types of infectious diseases and total chronic diseases patients present per HH were also tested and tabulated.

Results

Demographic characteristics

A total of 96 HHs from two districts (District I: Swat = 50, District II: Shangla = 46) participated voluntarily, resulting in an 88.8% response rate response rate was observed. Considering the cultural values in the study locations, only male members of the HHs took part in the study with the mean age group 18–28 ($n = 45$, 46.9%) years. The majority of its members had primary education ($n = 28$, 29.2%), were self-employed ($n = 32$, 33.3%), and ($n = 29$, 30.2%) with an average monthly income of PKR 21000–30000. Most of the families had six to eight members ($n = 32$, 33.3%), had family members with one or two chronic diseases ($n = 63$, 65.6%), were individual families (n=60, 62.5%), and had prevalence of LRTIs ($n = 35$, 36.5%). A small ($n =$ 14,14.6%) number of healthcare professionals were present at home (Table 1).

Antibiotic identification, knowledge, and ABR

The overall score for knowledge of antibiotic was relatively good and nearly half of the participants provided correct answers to the statements asked as presented in Table 2. The term antibiotics and knowledge related to antibiotics identification was about 57.3% ($n = 55$). But less than half of the respondents could correctly identify amoxicillin as an antibiotic. Additionally, a majority (61.5%) provided incorrect responses to the correct use of antibiotics and believed that ABs were efficacious in flu and cold. About half of the

TABLE 1 Demographic characteristics.

Demographic variables	*n* (%)
Location of the participants*	
District-I	50 (52.1)
District-II	46 (47.9)
Age of the participants (years)	
18–28	45 (46.9)
29–38	27 (28.1)
39–48	14 (14.6)
49–58	6 (6.3)
>60	4 (4.2)
Education level of the participants	
Primary education	28 (29.2)
Secondary education	18 (18.8)
Higher secondary education	15 (15.6)
Graduation	13 (13.5)
Postgraduation	4 (4.2)
No-formal education	18 (18.8)
Occupation of the participants	
Government employee	19 (19.8)
Private employee	23 (24)
Self-business	32 (33.3)
Daily wager	14 (14.6)
Other	8 (8.3)
Family set-up of the participants	
Individual	60 (62.5)
Joint family	35 (36.5)
Extended family	1 (1)
Total number of a family (Male and Female)	
2–4	4 (4.2)
4–6	27 (28.1)
6–8	32 (33.3)
10–12	21 (21.9)
>12	12 (12.5)
Average monthly income of the participants (PKR)	
1,000–20,000	9 (9.4)
21,000–30,000	29 (30.2)
31,000–40,000	21 (21.9)
41,000–50,000	9 (9.4)
>50,000	28 (29.2)
Total number of patients with chronic diseases	
0	33 (34.4)
1–2	63 (65.6)
Presence of the infectious/ other diseases	
Lower respiratory tract infections (LRTIs)	35 (36.5)
Upper respiratory tract infections (URTIs)	11 (11.5)
Other infectious disease (TB)	13 (13.5)
Chronic illness (Hypertension; Diabetes mellitus)	29 (30.2)
Not any disease (NAD)	8 (8.3)
Presence of healthcare professionals at home	
Yes	14 (14.6)
No	82 (85.4)

*Study Locations (District-I=Swat; District-II=Shangla).

participants answered the side effects statements and reported that antibiotics kill useful bacteria and ABs can cause (67.7%) of allergic reactions. However, they were unaware of ABR as just 28.1% expressed little knowledge about drug resistance (Supplementary Tables 1–4).

The prototype that predicted strong antibiotic knowledge and AMR found multiple meaningful associations. Each level of education, from elementary school to graduation, was strongly linked to superior knowledge ($p < 0.05$). Knowledge also had a relationship with age group ($p < 0.05$), occupation ($p < 0.05$), and family income ($p < 0.05$), all of which have been proven to be favorable to the knowledge of antibiotics due to their level of education (Table 5). The type of family structure, the number of family members, and the type of infectious disease were not significant indicators of antibiotic use or knowledge of AMR (Supplementary Tables 1–4).

HHs antibiotics knowledge

HHs antibiotics identification

The households were ($n = 55$, 57.3%) aware of the term "antibiotic" and that such medicines can fight against infectious diseases. Participants reported correct responses related to ($n = 42$, 43.8%) amoxicillin as an antibiotic, and more than half ($n = 50$, 52.1%) identified paracetamol as not an antibiotic (Supplementary Table 1). Knowledge related to the "other than antibiotics" category was recognized as antibiotics, and a very low number responded correctly ($n = 38$, 39.6%). The Median (IQR) Knowledge score regarding the identification of antibiotics was 2 (1–3) (Table 2).

HHs antibiotics use

Most of the HHs believed that antibiotics killed the germs ($n = 52$, 54.2%), and only half ($n = 48$, 50%) knew "good bacteria" was present in everybody's bodies (Supplementary Table 2). Despite the frequent use of antibiotics, more than half of the HHs ($n = 65$, 67.7%) provided accurate information regarding allergic reactions as HHs experienced such allergic reactions. They reported numerous instances of adverse reactions caused by antibiotics.

TABLE 2 Knowledge assessment regarding antibiotics and correct responses of the participants.

Knowledge regarding the identification of antibiotics	CR* *n* (%)
Have you ever heard of a type of medicine called antibiotics?	56 (58.3)
Is amoxicillin an antibiotic?	42 (43.8)
Is paracetamol an antibiotic?	51 (53.1)
Is aluminum hydroxide + magnesium hydroxide (antacid) an antibiotic?	38 (39.6)
Knowledge regarding the uses of antibiotics	
Are antibiotics useful for killing germs?	52 (54.2)
Are antibiotics often needed for cold and flu illnesses?	18 (18.8)
Does diarrhea get better faster with antibiotics?	59 (61.5)
Can antibiotics kill "good bacteria" present in our bodies?	48 (50)
Can antibiotics cause secondary infections after killing good bacteria present in our bodies?	46 (47.9)
Can antibiotics cause allergic reactions?	65 (67.7)
Knowledge regarding ABR	
If bacteria are resistant to antibiotics, it can be very difficult to treat the infections they cause.	27 (28.1)
Did you hear the term ABR?	26 (27.1)
Storage of unnecessary antibiotics is one of the reasons for ABR.	30 (31.3)
If bacteria are resistant to antibiotics, it can be very difficult to treat the infections they cause.	35 (36.5)
Many infections are becoming increasingly resistant to treatment by antibiotics.	34 (35.4)
Misuse of antibiotics can lead to ABR.	35 (35.5)
Knowledge score	**Median (IQR)**
Knowledge regarding identification of antibiotics	2 (1–3)
Knowledge regarding uses of antibiotics	4 (2–5)
Knowledge regarding ABR	1 (0–3)

*CR, Correct Responses.

HHs knowledge related to ABR

The majority of the HHs (n = 70, 72.9%) had not heard the term ABR before and they showed less awareness and knowledge about ABR. As evident from their responses, many of them provided incorrect answers about the treatment after bacteria/germs get resistant to the antibiotics; 45.8% (n = 44) responded with "don't know" about such incidents (Supplementary Table 4).

Respondents believed that the storage of unnecessary antibiotics is not a cause for ABR and only 31.3% (n=30), answered correctly. The Median (IQR) Knowledge score regarding ABR was 1(0–3). A majority of the respondents also had misconceptions regarding the misuse of antibiotics and it was common among HHs (n = 35, 35.5%) (see Table 2).

Overall understanding of antibiotics use, ABR, and storage of antibiotics in households

Likert scale responses

The Likert scale was used to measure the responses from HHs ranging from "strongly agree" to "strongly disagree." Less than half of the participants (n = 33, 34.4%) agreed that antibiotics should be identified alongside other medications, even if they are prescribed separately. The urinary and respiratory tract infections can be treated only with antibiotics (n = 34, 35.4%) and just a small proportion of HHs had such knowledge. Only 25 HHs strongly agreed with the statement regarding penicillin-triggered allergic reactions and the test dose (n = 35, 36.5%). The majority of HHs (n = 50, 52.1%) were unconcerned about the statement that ABR is caused by antibiotics failing to kill or inhibit bacterial growth. Within households, family members shared antibiotics and less than half of HHs (n = 29, 30.2 %) agreed to the prospect of ABR owing to an incomplete antibiotic course. ABR can develop due to incomplete doses (as a result of HHs storing antibiotics for future illnesses), and the majority of HHs remained indifferent (n = 33, 34.4%), with fewer than half (n = 29, 30.2%) agreeing with the assertion on ABR (refer Table 3).

HHs antibiotics storage

Antibiotics were stored at home by the majority of HH respondents (n = 80, 83.3 %) together with any other drugs for multiple purposes. Most of them were willing to show their stored antibiotics (n = 94, 97.9%), and a significant proportion of the antibiotics (n = 93, 96.9%) were in the oral dosage form. Antibiotics used for present illness by HHs (n = 50, 52.1%), were also used by other HH members (n = 46, 48.9%), and/or retained for future use. The antibiotics used by the majority of the HHs (n = 88, 91.7 %) for their current illness were purchased from the pharmacy/drug store staff (n = 41, 42.7 %). Antibiotics were available without prescription and only a small number (n = 23, 24%) of HHs got their antibiotics through prescription. Nearly half of the HHs (n = 47, 49%) received entire strips of antibiotics, more than the exact number required for the antibiotic course (refer to Table 4).

Antibiotics at HHs

Azithromycin (J01FA10) was the most stored antibiotic (n = 38, 39.6%) under different brand names. The second most stored antibiotic was "amoxicillin along with clavulanic acid" (J01CR), a common brand of the multinational pharmaceutical industry (n = 30, 31.2%), and "amoxicillin" (J01CA04) (n = 18, 18.7%). The fluoroquinolones group "ciprofloxacin" (J01MA02) was found as the third most reported (n = 24,

TABLE 3 Overall HHs knowledge related to antibiotics use, ABR, and infectious diseases.

Questions	LS*	*n* (%)
I can recognize antibiotics in my prescription as I always differentiate antibiotics from other medicines.	SA	14 (14.6)
	AG	33 (34.4)
	NT	24 (25)
	DA	22 (22.9)
	SDA	3 (3.1)
LRTIs, URTIs, and UTIs are only treated by antibiotics.	SA	11 (11.5)
	AG	34 (35.4)
	NT	34 (35.4)
	DA	13 (13.5)
	SDA	4 (4.2)
Antibiotics such as penicillin can cause allergic reactions, if not checked with the patient with the test dose.	SA	25 (26)
	AG	35 (36.5)
	NT	24 (25)
	DA	10 (10.4)
	SDA	2 (2.1)
ABR is referred to the inability of antibiotics to kill/inhibit microorganisms' growth.	SA	23 (24)
	AG	22 (22.9)
	NT	50 (52.1)
	DA	1 (1)
	SDA	0 (0)
HH storage of antibiotics for future illnesses can lead to ABR.	SA	20 (20.8)
	AG	29 (30.2)
	NT	33 (34.4)
	DA	12 (12.5)
	SDA	2 (2.1)
Sharing of antibiotics in HH members can develop ABR.	SA	12 (12.5)
	AG	29 (30.2)
	NT	48 (50)
	DA	6 (6.3)
	SDA	1 (1)
Self-medication with antibiotics is one of the reasons for ABR.	SA	18 (18.8)
	AG	23 (24)
	NT	46 (47.9)
	DA	8 (8.3)
	SDA	1 (1)
Consultation with a physician for the (RTIs and UTIs) illness and pharmacist counseling can enhance the appropriate use of antibiotics.	SA	15 (15.6)
	AG	22 (22.9)
	NT	56 (58.3)
	DA	1 (1)
	SDA	2 (2.1)

*LS, Likert Scale (SA, strongly agree; AG, agree; NT, neutral; DA, disagree; and SDA, strongly disagree).

25%) antibiotic among HHs. Third-generation cephalosporin "ceftriaxone" (J01DD04) which is common in rural areas was reported the fourth (n=23, 24%) most stored antibiotic among HHs, (n = 12, 12.5%) followed by "cefixime" (J01DD08).

TABLE 4 HH storage of medications (antibiotics).

Storage of medications	*N* (%)
Do you currently keep antibiotics at home?	
Yes	80 (83.3)
No	16 (16.6)
If yes, are you willing to show it/them to me?	
Yes	94 (97.9)
No	2 (2.1)
Dosage form	
Oral	93 (96.9)
Other	3 (2.9)
Status of antibiotics	
On use (first user)	50 (52.1)
On use (another person and for future use)	46 (48.9)
Purpose of antibiotic use?	
For current illness	88 (91.7)
For future illness	3 (3.1)
Don't remember	3 (3.1)
For emergency use	2 (2.1)
Who advised you to get the medicine?	
Physician	52 (54.2)
Pharmacy/drug outlet staff	41 (42.7)
Family/relatives/friends	3 (3.1)
How have you got antibiotics?	
With prescription	73 (76)
Without prescription	23 (24)
From where you have got antibiotics? (Source)	
Pharmacy	21 (21.9)
Drug store (small outlet)	75 (78.1)
When/How long has it been?	
For the last 10 days	36 (37.5)
For last 20 days	35 (36.5)
From last month	17 (17.7)
More than 1 month	8 (8.3)
Why was this source chosen?	
Easy access	75 (78.1)
Due to need	21 (21.9)
Type of package?	
Blister pack	32 (33.3)
Strip pack	47 (49)
Bottle	15 (15.6)
Bottle and Strip	2 (2.1)
Storage place	
Open place	5 (5.2)
Cabinet	6 (6.3)
Room	85 (88.5)
Antibiotics name	
J01CA (penicillin)	11 (11.5)
J01CA04 (amoxicillin)	18 (18.7)

(Continued)

TABLE 4 (Continued)

Storage of medications	*N* (%)
J01CR (amoxicillin + clavulanic acid)	30 (31.2)
J01FA10 (azithromycin)	38 (39.6)
J01FA09 (clarithromycin)	6 (6.3)
J01FA01(erythromycin)	6 (6.3)
J01AA07 (tetracycline)	8 (8.3)
J01AA02 (doxycycline)	13 (13.5)
J01MA02 (ciprofloxacin)	24 (25)
J01MA12 (levofloxacin)	12 (12.5)
J01MA01(ofloxacin)	13 (13.5)
J01DB05 (cefadroxil)	5 (5.2)
J01DD04 (ceftriaxone)	23 (24)
J01DD08 (cefixime)	12 (12.5)
J01FF01 (clindamycin)	2 (2.1)
J01XA01 (vancomycin)	6 (6.3)
J01XD01 (metronidazole)	31 (32.3)
More than one antibiotic	
J01MA02 + J01XD01	19 (19.8)
J01CA04+ J01FA09 + J01MA12 + J01FA10 + J01XD01	14 (14.6)
Number of unnecessary HH storage of antibiotics	
1–2	62 (64.6)
3–4	29 (30.2)
5–6	5 (5.2)

Few HHs also had "levofloxacin" (J01MA12) and ofloxacin (J01MA01) (n = 12, 12.5% and n = 13, 13.5%) respectively. Metronidazole (J01XD01) use was found concurrently (n=31, 32.3%) with other antibiotics to treat various infectious diseases. Many HHs stored more than one antibiotic in combinations. They had several unnecessary 1–2 (n = 62, 64.6%) and 3–4 (n = 29, 30.2%) antibiotics at their homes (Table 4). Figure 1 shows the storage of antibiotics at home for various infectious diseases based on the total number of its members. Except for two to four family members, who had a significant correlation with antibiotic storage ($p < 0.05$) and maintained fewer antibiotics at home than the other family members, the cross-tabulations reveal that the number of family members had no bearing on how antibiotics were stored (Figure 1).

Comparison of HHs demographic characteristics and antibiotics uses and storage

Various demographic parameters were checked against antibiotics knowledge and use scores. Different age groups had a strong association ($p = 0.017$, H = 12) and affected the mean knowledge scores related to antibiotic use. Education was one of the main factors that determined proper storage and use, and this study found a significant association (p=0.001, H= 52.8) between education and HHs' understanding of antibiotics. Similarly, employment had a direct effect on proper antibiotic use and a strong association ($p = 0.04$, H = 10) was observed between employment and HHs' perspectives. The family setup ($p > 0.05$, H = 0.7), the total number of family members ($p > 0.05$, H = 0.5), and monthly income ($p > 0.05$, H = 7.3) of HHs did not affect the understanding of antibiotics identification, use, and ABR and no relationship was found among the variables. Although, family members having more than one chronic disease ($p = 0.09$, H = 0.8) had a near significant relationship in their understanding of antibiotics and their uses ($p = 0.016$, U = 735) (see Table 5).

The overall results show that immediate attention is needed to enhance the rational use of antibiotics in households that do not have adequate knowledge of antibiotics, their identification, proper uses, and ABR-related factors.

Discussion

The current study is the first of its kind in Pakistan's post-conflict districts. The results of this survey provide an up-to-date picture of HHs and medicines storage (antibiotics), which will contribute to the development of community education initiatives to promote sensible antibiotic use. The purpose of this study was to examine the knowledge and habits of HHs on antibiotic use and storage. Instructing HHs on medicines and their storage is one of the most important aspects of antibiotic use after receiving them from the community pharmacy, as the rest of the therapy outcomes are dependent on their proper intake at home. Our study showed a significant lack of awareness regarding the proper use of antibiotics, ABR, and needless medication storage at home, which indicated poor antibiotic practices at HHs.

The accurate identification of antibiotics is essential. Knowledge gaps in antibiotics use can lead to ABR, and we observed such a lack of knowledge among HHs in the present study. We found that antibiotic identification was relatively poor (39.6%) and similar results have been reported by Atif and his colleagues, and Tawseef et al., in their studies (34, 35). This problem is not limited to Pakistan alone; researchers from other countries have undertaken similar studies, particularly among rural low and low middle-income countries (LMIC) communities with varying levels of knowledge ranging from moderate to poor, touch comparisons are difficult (32, 36–38). As a result, our findings, as well as those of other research that looked into antibiotic knowledge in greater depth, show that there is still a lot of work to be done in terms of educating rural populations in LMICs about antibiotics and the proper use (31, 39, 40). Comparable results have also been reported in China where knowledge gaps and unfavorable attitudes

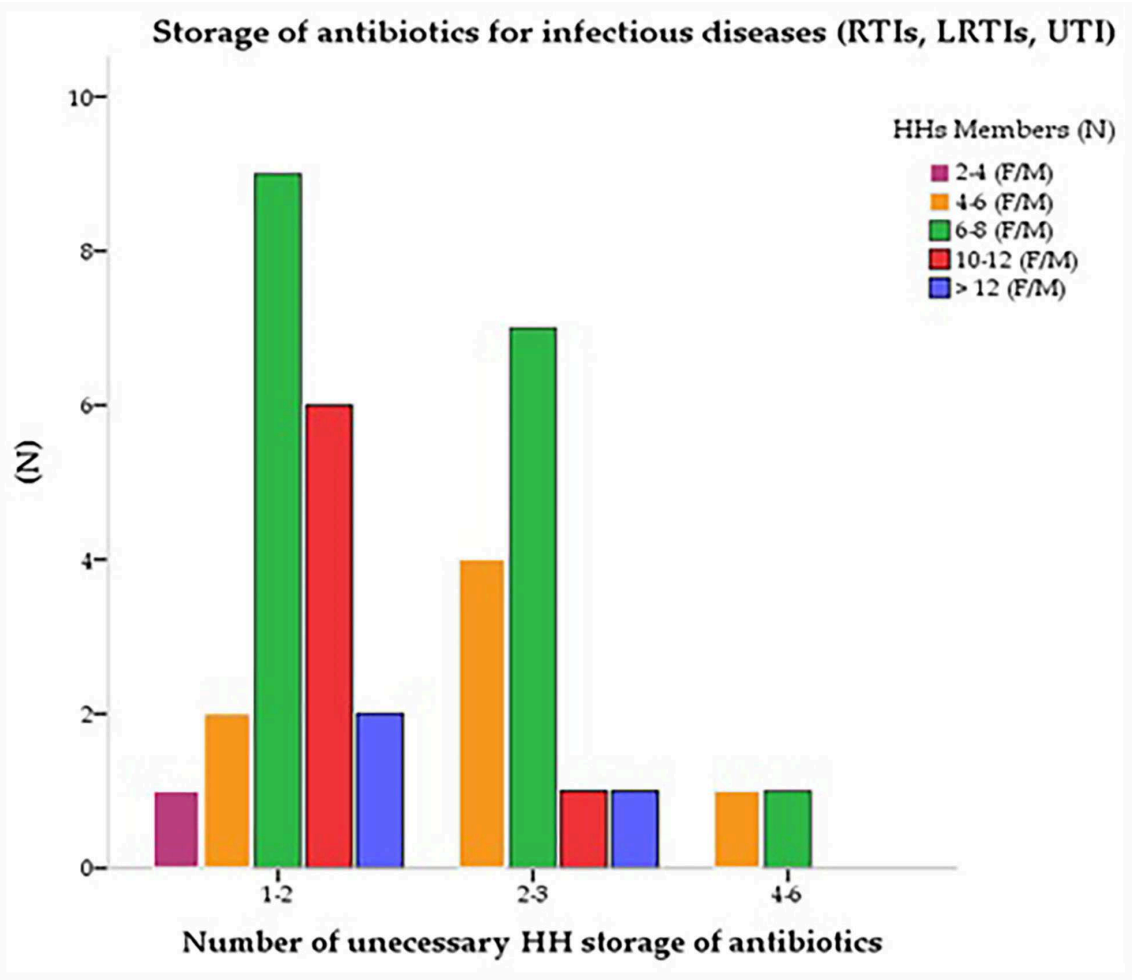

FIGURE 1
The total number of HHs vs. antibiotics storage for infectious diseases.

regarding the proper use of antibiotics were detected among HHs. About 48.2% of respondents said they had used antibiotics for children without a prescription (41). Antibiotic knowledge is also low in countries in the Middle East, especially in Saudi Arabia; according to a survey, a majority of respondents sought antibiotics without a prescription (42). According to the reported study, 71% of Saudis and 36% of Kuwaitis did not complete their antibiotic medication course (42, 43). Findings from Jordan were similar to our results, with 60.7% of individuals having an insufficient understanding of the antimicrobial resistance (44). Compared to its neighboring countries, antibiotic misuse in Qatar is extremely high (85%) (45). Patients took antibiotics without knowing it and were unfamiliar with the terms, particularly "ABR" and comparable findings have been reported from other countries as well.

One of the most important findings of the current study is that respondents incorrectly believed that they could stop taking antibiotics midway without completing the course; about 43% of the Serbian population also believes likewise (46). The situation is particularly bad in African regions, where an urgent call for work on an action plan against ABR is required to address the problem. Equally, India, Iran, and other Asian countries also need extensive attention (47–49). According to a comparable study from South Africa, a better level of education will boost antibiotic awareness and proper use among consumers (50, 51). To avoid ABR and inappropriate use, antibiotic dosing regimens must be finished within the timeframe given (five to seven days or as recommended). Summarizing the current situation in European countries A Machowska and C S Lundborg highlight that major factors driving ABR among the general public are lack of public knowledge and awareness, access to antibiotics without prescription, and leftover antibiotics at home (52). According to the latest Eurobarometer survey, 34% of Europeans used antibiotics at least once in 2016 (52). Antimicrobial/antibacterial

TABLE 5 Comparison of demographic characteristics with respect to knowledge regarding the identification of antibiotics.

Variables	Groups	*N*	Mean Rank	H	*p*-value*
Age	18–28	45	50.42	12	0.017
	29–38	27	58.52		
	39–48	14	38.11		
	49–58	6	25.67		
	>60	4	29.88		
Education level of the participants	Primary education	28	32.77	52.8	0.001
	Secondary education	18	58.56		
	Higher secondary education	15	64.37		
	Graduation	13	80.96		
	Postgraduation	4	64.38		
	No formal education	18	22.72		
Occupation of the participants	Govt employee	19	61.42	10	0.04
	Private employee	23	45.15		
	Self-business	32	41.94		
	Daily wager	14	42.21		
	Other	8	64.69		
Family set-up of the participants	Individual	60	49.58	0.7	0.7
	Joint family	35	47.23		
	Extended family	1	28.5		
Total number of family (male and female)	2–4	4	55.75	0.5	0.9
	4–6	27	49.83		
	6–8	32	46.53		
	10–12	21	47.88		
	>12	12	49.42		
Average Monthly Income of the Participants (PKR)	1,000–20,000	9	56.11	7.3	0.1
	21,000–30,000	29	44.29		
	31,000–40,000	21	37.98		
	41,000–50,000	9	58		
	> 50,000	28	55.25		
Type of the infectious diseases	Lower respiratory tract infections	35	44.27	8	0.09
	Upper respiratory tract infections	11	35.05		
	Other infectious diseases (TB, etc.)	13	59.15		
	Chronic illnesses	29	55.72		
	Not any disease (NAD)	8	42		
Number of chronic diseases	**Groups**	**N**	**Mean Rank**	**U**	***p*-value****
	0	33	57.73	735	0.016
	1–2	63	43.67		

$p \leq 0.05$ was considered statistically significant. *p-value was calculated using the Kruskal Wallis H test. **p-value was calculated using the Mann Whitney U-test.

resistance is not only an issue in developing countries but also a major problem in developed countries. All governments and regions must investigate the irrational use of antimicrobials to establish effective strategies to combat antimicrobial resistance (51, 53). Our results could be related to those from other low and middle-income countries (38), as well as Germany, the United Kingdom, Sweden, Italy, Poland, Lithuania, Cyprus, Siberia, and Hong Kong. The present finding emphasized the appropriate use of antibiotics among HHs and discourage the unnecessary storage of antibiotics through patients' education and awareness in the community.

In the current study, the majority of customers preferred to store unnecessary antibiotics for future usage, and near similar findings have been reported from China (31%) and

Jordan (49%) (44, 54). The most common way to access nonprescription antibiotics is through leftover medicines. Non-prescription antibiotic use among children in HHs was found to be significantly linked to antibiotic storage at home (41). Moreover, more than half of the total HHs in this study were willing to keep antibiotics at home, which is a little different from studies in Indonesia, Iran, Iraq, Oman, Greece, and the United States, where 82 to 100% percent of HHs stored antibiotics at home (17). When it came to keeping antibiotics or other medicines, families with members who had chronic illnesses were more inclined to do so than those who did not experience such illness. The likelihood of antibiotics storage was also associated with HHs occupation. Other research studies have come to similar conclusions (2, 17, 55). Antibiotics that have been left over should be disposed of correctly. Other countries' experiences can be beneficial to Pakistan. Residents of Portugal, Sweden, and the Netherlands, for example, regularly return unused antibiotics to the pharmacy (30, 56). The Starfish Project in the United States gathers unwanted antibiotics from HHs through the use of free mail labels (30). While a lack of public awareness about inappropriate antibiotic use in self-limiting diseases contributes to the high prevalence of over-the-counter antibiotic purchases, easy access to over-the-counter antibiotics is also a factor in leftover antibiotics as reported in similar other studies (30, 57). According to Mutaseim Makki and his colleagues, the incidence of unused prescriptions in HHs has grown drastically in recent decades, resulting in medicines wastage (58). Patients' non-adherence to medication-taking was shown to account for 50% of unnecessary storage which leads to wastage (58).

According to studies, areas that have experienced continuous conflict are prime locations for bacteria to develop resistance and misuse of antibiotics (59). Violent conflict and displacement heighten the difficulties of limiting the emergence and spread of AMR. LMICs affected by conflict must not only deal with resource constraints but also discover ways to care for and treat injured people carrying more resistant infections (60). According to the literature and expert opinions, better lab diagnoses, the establishment of surveillance systems, and infection prevention and control should be prioritized along with AMR control strategies in the conflict-affected LMICs (61). However, there is a lack of research on the usefulness of these strategies in such conflict situations. More research studies are needed in post-war zones so that effective treatments can be devised and implemented.

Public awareness efforts and awareness campaigns are needed to discourage people from using leftover antibiotics at home for themselves or their families and to encourage people to properly dispose of leftover medicines. Our findings show that personalized interventions can reach people with a wide range of demographic and socioeconomic factors. Family members with a history of infectious diseases like respiratory tract infections were a significant risk factor for keeping antibiotics at home, according to our findings (43, 44).

"World Antibiotic Awareness Week" was organized by the National Institute of Health (NIH), Pakistan, on 18–24 November 2021. Such occasions can result in a step-up in Pakistani society's consciousness. The NIH must stress the significance of not keeping antibiotics at home and educate HHs in the affected areas. Therefore, now is an excellent time (spring) to launch a program for HHs and communities, particularly in Pakistan's post-conflict rural areas, to inform the people about the proper use of antibiotics and the adverse effects (ABR) of inappropriate antibiotics consumption. The findings revealed by Khan et al. (57), are very comparable to the current research conducted in post-conflict regions (5). The consumer-related factors were evaluated at community pharmacies through quantitative and qualitative approaches (38).

There are a few limitations that should be considered while interpreting the results of our study. First, it was a cross-sectional study of Pakistan's post-conflict areas that focused on the most impacted districts from militancy and army operations. It did not cover the rest of the Khyber Pakhtunkhwa, Malakand division districts; thus, the findings cannot be generalized. However, these results can be considered for similar other conflict areas in the country. Second, due to cultural norms, women's participation was not documented, as the men always represent their families during any visit to the HHs in these areas; all these limitations are an integral part of the rural areas of Swat, Khyber Pakhtunkhwa customs, and traditions. However, we believe that female participation will not largely impact the results as these regions are primarily led by men who are decision-makers in their families and a convenient sampling method was employed. Third, the sample was taken from only two sites and the sample size is small. Fourth, we only included 'antibiotics' among the stored medications at home, no additional medications were stated by the HHs. In Pakistan's rural areas, more research on medicines storage among HHs is required.

Conclusion

This study showed that storage of antibiotics for future use is quite common among HHs residing in post-conflict regions of Pakistan. Households identified antibiotics among the stored drugs based on packaging and several common brands but were unable to explain why leftover antibiotics should be saved for future use as the storage of antibiotics was observed. The majority of HHs were not aware of ABR and did not explain how antibiotics cause resistance. Furthermore, ABR is the result of irrational antibiotic use, and antimicrobial/antibiotic oversight measures are still weak in Pakistan. The ABR and antibiotic stewardship are still unknown to the rural population. In terms of healthcare, Pakistan's post-conflict areas are the most

neglected. More research studies are needed to raise awareness of the ABR problem and to educate HHs on how to use antibiotics properly when they are needed.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and the Ethics Committee for Medical Research at Xi'an Jiaotong University has given its approval to our study (Ref.No: 2020-1342). The patients/participants provided their written informed consent to participate in this study.

Author contributions

FaiK and YF: the study concept and design. FaiK: writing of the manuscript. QK: data analysis. TA and FarK: obtaining data. TM and KH: interpretation of results. TM and YK: critical revisions of the manuscript. All authors approved of the version for submission.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.962657/full#supplementary-material

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The efficacy and safety of eravacycline compared with current clinically common antibiotics in the treatment of adults with complicated intra-abdominal infections: A Bayesian network meta-analysis

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Background: Eravacycline is a novel, fully synthetic fluorocycline antibiotic for the treatment of adults with complicated intra-abdominal infections (cIAIs). However, the efficacy and safety of eravacycline compared with current clinically common antibiotics remain unknown.

Objective: This study aims to compare the efficacy and safety of eravacycline and other clinically common antibiotics in China, including tigecycline, meropenem, ertapenem, ceftazidime/avibactam+metronidazole, piperacillin/tazobactam, imipenem/cilastatin, and ceftriaxone+metronidazole, for the treatment of adults with cIAIs and to provide a reference for clinical choice.

Methods: The PubMed, Embase, Cochrane Library, and ClinicalTrials.gov databases were electronically searched to collect clinical randomized controlled studies (RCTs) comparing different antibiotics in the treatment of patients with cIAIs from inception to June 1, 2021. Two reviewers independently screened the literature, extracted data, and evaluated the risk of bias in the included studies.

Results: A total of 4050 articles were initially retrieved, and 25 RCTs were included after screening, involving eight treatment therapies and 9372 patients. The results of network meta-analysis showed that in the intention-to-treat (ITT) population, the clinically evaluable (CE) population, and the microbiologically evaluable (ME) population, the clinical response rate of eravacycline was not significantly different from that of the other 7 therapies ($P > 0.05$). In terms of microbiological response rate, eravacycline was significantly better than tigecycline [tigecycline vs. eravacycline: RR = 0.82, 95%CI (0.65,0.99)], and there was no significant difference between the other 6 regimens and eravacycline ($P > 0.05$). In terms of safety, the incidence

of serious adverse events, discontinuation rate, and all-cause mortality of eravacycline were not significantly different from those of the other 7 treatment therapies ($P > 0.05$).

Conclusion: Based on the evidence generated by the current noninferiority clinical trial design, the efficacy and safety of eravacycline for the treatment of adults with cIAIs are not significantly different from those of the other 7 commonly used clinical antibiotics in China. In terms of microbiological response rate, eravacycline was significantly better than tigecycline. In view of the severe multidrug-resistant situation in China, existing drugs have difficulty meeting the needs of clinical treatment, and the new antibacterial drug eravacycline may be one of the preferred options for the treatment of cIAIs in adults.

KEYWORDS

complicated intra-abdominal infections, eravacycline, randomized controlled trials, systematic review, network meta-analysis

Introduction

Complicated intra-abdominal infections (cIAIs) in adults originate from abdominal organs and spread to the peritoneal or retroperitoneal space, causing peritonitis and abdominal abscesses. Intra-abdominal infections are the most common infectious disease among hospitalized patients (1–3), and the mortality rate varies greatly due to different factors, such as disease severity and the range of infecting pathogens. The mortality rate in patients with hospital-acquired cIAIs is significantly higher than that of community-acquired infections (10.4 vs. 2.8%) in China, and a higher mortality rate is prevalent in patients with cIAIs in the intensive care unit (ICU) (21.24–29.1%) (4–8). cIAIs can be combined with complications such as sepsis, septic shock, and multiple organ failure. Surgery combined with antibiotics is generally recommended as treatment in clinical practice (1).

Over the past century, great strides have been made to treat cIAIs with various drugs. However, with the widespread application of antibacterial drugs in clinical practice, especially their irregular use or overuse, antimicrobial resistance among pathogens continues to increase. Therefore, multidrug-resistant bacteria have become an important threat to human health around the world. The clinical efficacy of antibacterial drugs such as penicillin, cephalosporin and carbapenems in the treatment of cIAIs has been seriously affected in recent years due to the increasing drug resistance of carbapenem-resistant Enterobacteriaceae or bacteria that produce extended-spectrum-β-lactamases (ESBLs) (9). Studies have shown that the first-line empirical treatment failure rate of cIAIs is as high as 68.3% (10). Moreover, infections with multidrug-resistant bacteria will lead to a longer hospital stay and increase the risk of death for cIAI patients, which not only causes more serious damage to their health but also places a relatively heavy economic burden on their families and society (3, 11). For treatment of hospital-acquired and high-risk community-acquired cIAIs, the current commonly used antibiotics in China mainly include tigecycline, meropenem, ertapenem, ceftazidime/avibactam+metronidazole, piperacillin/tazobactam, imipenem/cilastatin, ceftriaxone+metronidazole and other monotherapies or combination therapies.

Eravacycline is a novel, fully synthetic fluorocycline antibiotic that has a broad antibacterial spectrum and can cover all common clinical pathogens except for *Pseudomonas aeruginosa*, including gram-negative and gram-positive aerobic and anaerobic strains (9, 12, 13). Furthermore, eravacycline show high sensitivity to drug-resistant bacteria, and it can therefore be used to treat infection with multidrug-resistant bacteria, such as bacteria producing ESBLs, carbapenem-resistant Enterobacteriaceae, and carbapenem-resistant *Acinetobacter baumannii*. The results of two phase III multicentre clinical randomized controlled trials (RCTs), IGNITE 1 and IGNITE 4 (12, 13), showed that the efficacy of eravacycline was not inferior to ertapenem and meropenem in patients with cIAIs. Based on this, eravacycline was approved by the United States and the European Union in 2018 for the treatment of adults with cIAIs. Although current evidence shows that the efficacy and safety of eravacycline are equivalent when compared with ertapenem and meropenem, considering that there are more treatment options for antibacterial drugs used in the clinical treatment of cIAIs in China and that we lack head-to-head clinical RCTs of these different antibacterial drugs, this study used a Bayesian network meta-analysis to compare the efficacy and safety of eravacycline and other clinically common antibiotics in the treatment of adults with cIAIs to

provide evidentiary support and a reference for rational clinical drug use in China.

Materials and methods

This study was conducted and performed in accordance with Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) guidelines (Supplementary Table 1) (14).

Literature search strategy

All clinical studies comparing the different antibiotics in the treatment of patients with cIAIs were identified through a systematic review of the literature in the PubMed, Embase, Cochrane Library, and ClinicalTrials.gov databases from inception to June 1, 2021. The following search terms were used: complicated intra-abdominal infection, clinical trial, randomized, efficacy, safety, eravacycline, tigecycline, ertapenem, meropenem, and ceftazidime/avibactam, among others. The reference lists of all retrieved articles were also reviewed to identify additional articles. The retrieval was taken in the form of a combination of subject words and free words. The final database-specific searches are presented in Supplementary Tables 2–5.

Eligibility criteria

Studies meeting the following criteria were included: (1) type of the study, RCT; (2) target population, adult patients with cIAIs; (3) intervention, novel treatment drug eravacycline; (4) comparison, current commonly used antibiotics in China, including tigecycline, meropenem, ertapenem, ceftazidime/avibactam+metronidazole, piperacillin/tazobactam, imipenem/cilastatin, and ceftriaxone+metronidazole; (5) outcome, the primary clinical efficacy endpoint of this meta-analysis was clinical response assessed at the test-of-cure (TOC) visit based on a modified/intention-to-treat (ITT) analysis population; the secondary clinical efficacy endpoint were clinical response assessed at the TOC based on clinical evaluable (CE) and microbiological evaluable (ME) populations. Microbiological efficacy endpoints were microbiological response at the TOC. Safety outcomes were all-cause mortality, any adverse events (AEs) leading to discontinuation and serious AEs ($\geq$ grade 3).

We excluded the following studies: (1) studies not in English; (2) duplicate studies; (3) systematic reviews, case observations, study protocols, lectures, conferences, and theses; (4) pooled analysis and post hoc analysis; (5) pharmacological, toxicology, molecular and animal experiments; (6) patients with any cancer; and (7) studies with no efficacy or safety outcomes.

Selection of studies and data extraction

Two reviewers independently screened the literature, extracted data, and cross-checked the data. The titles and abstracts were screened to exclude obviously irrelevant literature, and the full texts were further screened to determine whether they were finally included. Discrepancies were resolved by consensus. The following data were extracted from the studies: (1) first author/year of publication; (2) characteristics of the target population: number of patients, age, Acute Physiology and Chronic Health Evaluation (APACHE) score and severity of infection; (3) interventions and comparisons; (4) duration of treatment; (5) key elements to assess risk of bias; and (6) outcome measures.

Risk of bias assessment

We evaluated the methodological quality of the identified studies and cross-checked the results. The Cochrane Handbook for Systematic Reviews of Interventions version 5.1.0 was used to assess methodological quality. In terms of the assessment criteria, each trial was rated and assigned to one of the three following risks of bias: low risk, high risk or unclear. The evaluation items included random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and trial personnel (performance bias), blinding of assessor data (measurement bias), incomplete outcome data (follow-up bias), selective reporting of results (reporting bias) and other bias (15). Each evaluation item was divided into three levels: high risk of bias, low risk of bias and unclear risk.

Statistical analysis

Bayesian network meta-analysis was performed by using R 3.6.1 software (all code was present in Supplementary Table 6). The relative risk (RR) and 95%CI were used as effect analysis statistics for binary classification efficacy outcome endpoints, and the odds ratio (OR) and 95%CI were used as effect analysis statistics for binary classification safety outcome endpoints. When the network plot had a closed loop, the node analysis method was used to test the consistency of the direct comparison and indirect comparison results of the treatments of the loop. At a $p > 0.05$, the direct comparison result was considered to be consistent with the indirect comparison result, and the consistency model (CM) was used for the network meta-analysis; otherwise, the inconsistency model (IM) was used. Each model was initially set up with 4 Markov chains for simulation, and the number of iterations was 200,000 times. The first 20,000 annealing times eliminate the influence of the initial value, and the degree of model convergence is diagnosed by the Potential Scale Reduction Factors (PSRF), which indicates that

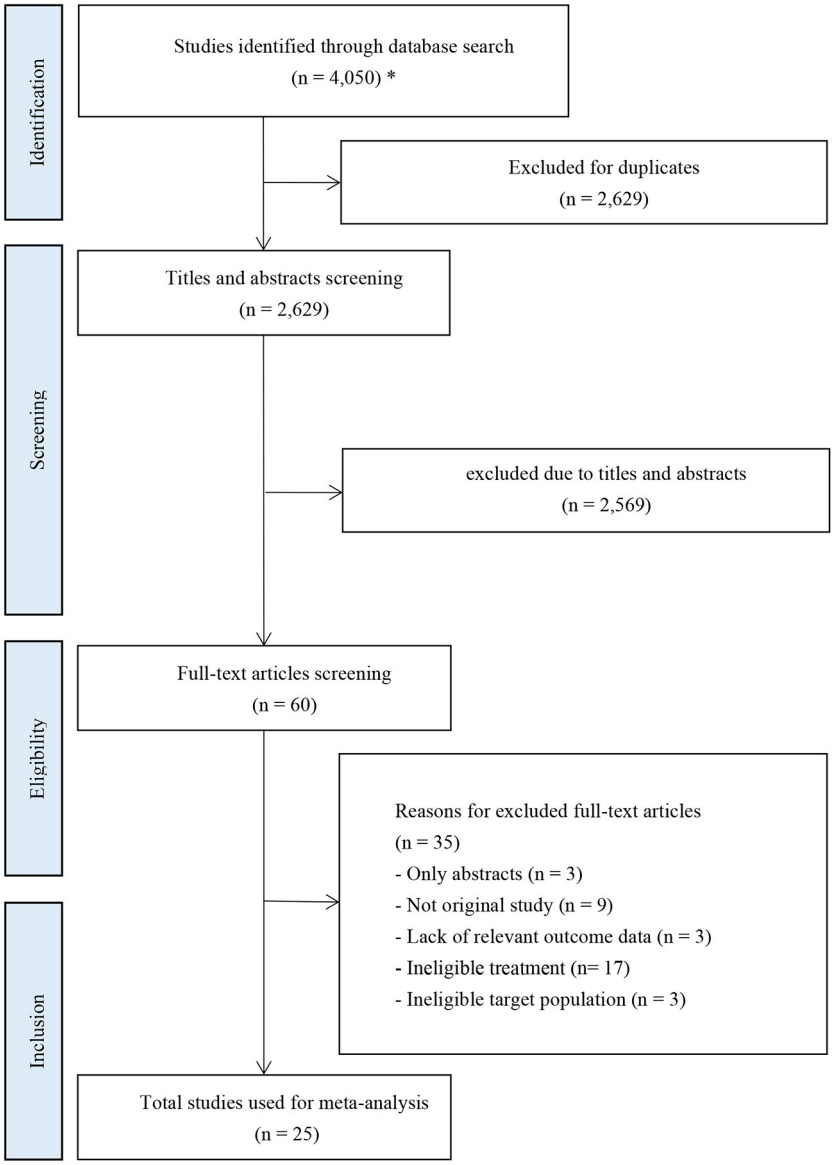

FIGURE 1
PRISMA flow diagram of study inclusion. *The databases searched and the number of records retrieved are as follows: PubMed (n = 1,328); Embase (n = 1,936); Cochrane Library (n = 761); ClinicalTrials.gov (n = 25).

the model convergence is satisfactory if its value is close to 1. The publication bias was evaluated by funnel plot and Begg's test.

Results

Selection process and outcomes for the studies

A total of 4,050 records were retrieved from the initial database search. Twenty-five RCT studies with a total of 9,372 patients were included after removing ineligible records. The selection process was shown in Figure 1.

Characteristics and summary of risk of bias of included studies

The basic characteristics of the included studies are listed in Table 1. Seventeen of the 25 RCTs were global multicentre studies. The assessment outcome of risk of bias is shown in Figures 2A,B. The figures show that 25 studies were of high

TABLE 1 Characteristics of included studies.

Author/year	Country/region	Numbers of patients (T/C)	Age (year) (T/C) (mean±SD)	APACHE II scores; (T/C) (mean ± SD)	APACHE II scores; (T vs C)(%)	Severity of infection	Intervention		Duration (Day) (T/C) (mean±SD)	Outcome endpoints
							Treatment	Comparator		
Basoli et al. (16)	20 centers from Italy	101/100	54.4	6.4/5.9	≤10: 80.2% vs 82.6%; 11-20: 19.8% vs 13%; >20: 0 vs 2%	Mild to moderate (not life-threatening)	Ipmcil	Mem	6.7/7.2	①②
Brismar et al. (17)	6 centers from Sweden	69/65	52.9/54	NA	NA	NA	Tzp	Ipmcil	5.5/5.9	①②③
Brismar et al. (18)	7 centers from Sweden	132/117	50.5/51.7	NA	≤10: 90.9% vs 89.7%; 11-20: 9.1% vs 9.4%; >20: 0 vs 0.9%	NA	Mem	Ipmcil	5/5	②③
Chen et al. (19)	47 centers from China	207/205	47.3 ± 17.74/8.7 ± 17.4	5.2 ± 3.38/5.4 ± 3.38	≤15: 100% vs 100%; >15: 0 vs 0%	NA	Tgc	Ipmcil	7.5/7.6	①②③④⑤
Chen et al. (20)	China	97/102	46.8 ± 18.2/41.0 ± 16.7	5.1 ± 3.9 /4.1 ± 2.7	NA	Mild to moderate	Tgc	Ipmcil	5/6	②③④⑤
Dela Pena et al. (21)	48 centers worldwide	180/190	48/49	2/2 (median)	>10: 3.9 vs 4.2%	NA	Etp	Tzp	4-14	③④⑤
Erasmo et al. (22)	China, Hong Kong, Malaysia, Korea, Philippines and Thailand	149/144	42.9 ± 18.3/41.3 ± 17.4	NA	NA	Moderate:65.4%; Severe:34.6%	Tzp	Ipmcil	5.6 ± 2.0/5.5 ± 2.1	①②④⑤

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TABLE 1 (Continued)

Author/year	Country/region	Numbers of patients (T/C)	Age (year) (T/C) (mean±SD)	APACHE II scores; (T/C) (mean ± SD)	APACHE II scores; (T vs C)(%)	Severity of infection	Intervention		Duration (Day) (T/C) (mean±SD)	Outcome endpoints
							Treatment	Comparator		
Fomin et al. (23)	94 centers from 27 countries in Europe, South Africa, Australia and Asia	404/413	48.3 ± 18.4/49.5 ± 18.0	6.44/ 6.41	NA	NA	Tgc	Ipmcil	7.7 ± 2.7/7.8 ± 2.7	①②③④⑤
Geroulanos et al. (24)	12 centers from 6 countries in Europe	116/116	55/54	NA	NA	Mild: 15.9%; Moderate: 59%; Severe: 24.6%	Mem	Ipmcil	7.8/8.3	①②③④
Kanellakopoulou et al. (25)	Greece	32/30	NA	NA	NA	Moderate:100%	Mem	Ipmcil	7.7/8.6	①②
Lucasti et al. (26)	33 centers from Bulgaria, France, India, Lebanon, Poland, Romania, Russia and America	101/102	43.0 ± 15.9/42.6 ± 18.1	NA	≤10: 83.2% vs 83.3%; 11–25: 16.8% vs 16.7%	Less severely ill	Cazavi	Mem	6/6.5	①②③⑤
Mazuski et al. (27)	136 centers from 30 countries	529/529	49.8 ±17.5/50.3 ± 18.3	NA	≤10: 84.0% vs 83.0%; 11–30: 15.0% vs 15.3%; >30: 0.2% vs 0	NA	Cazavi	Mem	8.0 ± 3.3/8.3 ± 3.1	①②③
Namias et al. (28)	61 centers from America	247/247	49.9/48.7	7.2 ± 4.3 / 6.4± 4.8	≤10: 83.7% vs 83.3%; >10: 16.3% vs 16.7%	NA	Etp	Tzp	7.0 ± 3.6/7.6 ± 4.0	①②③④
Navarro et al. (29)	53 centers from Latin America, Europe, Asia, Australia and South Africa	225/225	44/43.9	3/3 (median)	>10: 2.7% vs. 4.4%	NA	Etp	Ctrx	6/7	①②③④⑤

(Continued)

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TABLE 1 (Continued)

Author/year	Country/region	Numbers of patients (T/C)	Age (year) (T/C) (mean±SD)	APACHE II scores; (T/C) (mean ± SD)	APACHE II scores; (T vs C)(%)	Severity of infection	Intervention		Duration (Day) (T/C) (mean±SD)	Outcome endpoints
							Treatment	Comparator		
Oliva et al. (30)	Multicenter worldwide	247/255	42.9 ± 18.0/43.1 ± 17.6	5.6 /5.5	NA	NA	Tgc	Ipmcil	8.1 ± 2.8/7.9 ± 2.7	①②③④⑤
Qin et al. (31)	Centers from China, South Korea and Vietnam	214/217	48.5 ± 16.8/48.5 ± 17.4	NA	≤10: 93.9% vs. 92.6%; 11-30: 6.1% vs 7.4%	NA	Cazavi	Mem	6.9 ± 2.9/7.3 ± 2.8	①②③④⑤
Qvist (32)	Centers from 19 countries in Europe, Asia, South Africa and the Middle East	232/235	48.55 ± 18.37/46.81 ± 18.38	6.22 ± 4.02 / 6.99 ± 4.70	NA	NA	Tgc	Ctrx	6.97 ± 3.01/6.93 ± 2.71	①②③④⑤
Solomkin et al. (13)	66 centers from 11 countries	220/226	54.9 ± 17.14/55.4 ± 16.17	6.6 ±4.23 /6.8 ±3.94	NA	NA	Era	Etp	7.6 ± 2.8/7.6 ± 2.4	①②⑤
Solomkin et al. (33)	57 centers from 18 countries	323/193	46.2 ± 19.0/45.4 ± 18.9	NA	0–4: 29% vs 28%; 5–9: 41% vs 46%; 10–14: 21% vs 18%; 15–19: 6% vs 6%; 20–24: 2% vs 1%; 25–29: 0.5% vs 0	NA	Etp	Tzp		①②③④⑤
Solomkin et al. (12)	19 centers from 6 countries	57/30	42.1 ± 17.2/41.8 ± 17.6	6.0 ±3.8/6.1±2.7	NA	NA	Era	Etp	6.3/6.2	①②③④⑤
Solomkin et al. (9)	65 centers from 11 countries	195/205	50.3 ± 17.7/52.3 ± 18.3	6.6 ± 3.8 / 6.4 ± 4.0	NA	NA	Era	Mem	4-14	①②③④

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TABLE 1 (Continued)

Author/year	Country/region	Numbers of patients (T/C)	Age (year) (T/C) (mean±SD)	APACHE II scores; (T/C) (mean ± SD)	APACHE II scores; (T vs C)(%)	Severity of infection	Intervention		Duration (Day) (T/C) (mean±SD)	Outcome endpoints
							Treatment	Comparator		
Tellado et al. (34)	Multi-center worldwide	323/310	46.2 ± 19.0/45.4 ± 18.9	NA	0–4: 29.4% vs 29.6 %; 5–9: 41.1% vs 43.3%; 10–14:19.0% vs 18.6%; 15–19: 6.3% vs 7.2%	NA	Etp	Tzp	6/7	①②⑤
Towfigh et al. (35)	53 centers from America, Canada and Latin America	236/231	48/48	NA	<10: 80% vs 81% 10–15: 16% vs 15% >15: 4% vs 4%	NA	Tgc	Ctrx	4-14	①②③④⑤
Yellin et al. (36)	19 centers from America and Latin America	59/55	37.8 ± 18.1/41.1 ± 19.0	NA	0–4: 36% vs20% 5–9: 39% vs 56%; 10–14: 19% vs 18%; 15–19: 5% vs 2%; 20–24: 0% vs 2%	Mild to moderate	Etp	Ctrx	7.7 ± 4.3/8.8 ± 5.0	①②③
Zanetti et al. (37)	Centers from Sweden	71/64	59.8 ± 18.5/60.0 ± 18.6	5.8 ±3.5/ 6.4 ±4.2	0–5: 48% vs 47%; 6–10: 41% vs 39%; 11–15: 10% vs 8%; 16–18: 1% vs 6%	Moderate	Mem	Ipmcil	9.5 ± 3.6/8.4 ± 2.9	①②③

NA, not applicable; SD, standard deviation; Era, eravacycline; Etp, ertapenem; Mem, meropenem; Tgc, tigecycline; Cazavi, ceftazidime/avibactam + metronidazole; Tzp, piperacillin/tazobactam; Ctrx, ceftriaxone + metronidazole; Ipmcil, imipenem/cilastatin; ①Clinical response; ②Microbiological response; ③Mortality; ④any drug-related adverse events(AEs) leading to discontinuation; ⑤serious AEs(≥ grade 3).

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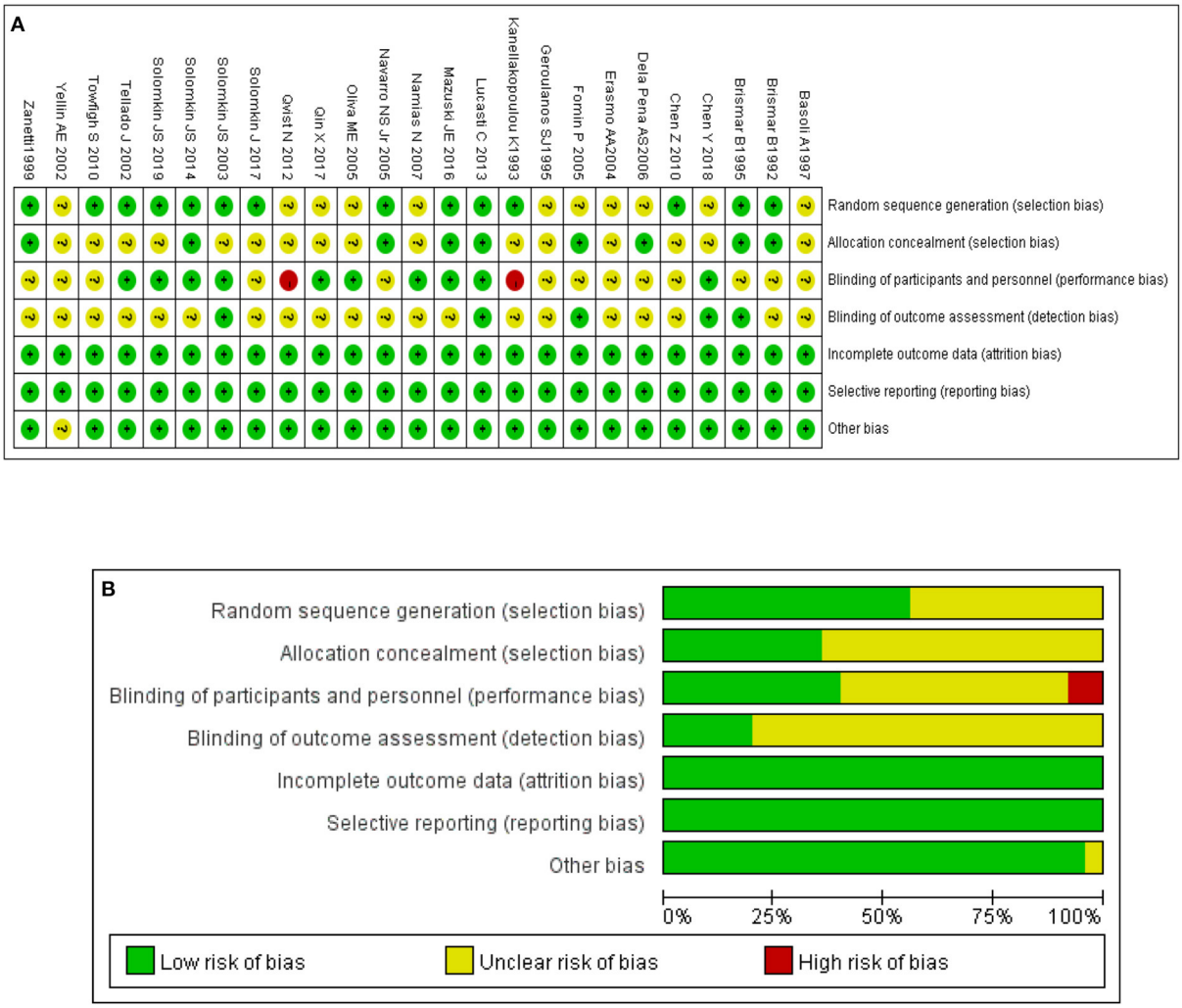

FIGURE 2
(A) Risk of bias summary. **(B)** Bar chart of risk of bias.

overall quality, with 23 having a low or unclear risk of bias and two studies possibly having a risk of selection bias (16, 17).

Convergence assessment and inconsistency test

The PSRF of the network meta-analysis model established according to all outcome indicators was close to 1, indicating that the model converged well and the outcomes were reliable. Since there were closed loops in the network diagrams of all outcome indicators, the node analysis method was used for the inconsistency test. The results showed no substantial difference ($P > 0.05$) between the direct comparison and indirect comparison of interventions in the loop, which met the requirements of consistency. Therefore, this study applied a consistency model to conduct a network meta-analysis of all outcome indicators.

Network meta-analysis for clinical response

A total of 4,024 ITT patients in the 10 RCTs provided data for clinical response evaluation. The network plots are shown in Figure 3, the thicker the line in the plots is, the more direct comparative studies between the two interventions are. Eravacycline showed no significant differences compared with the other seven treatments. However, eravacycline achieved a higher absolute clinical response rate than tigecycline [Tgc vs. Era: RR =

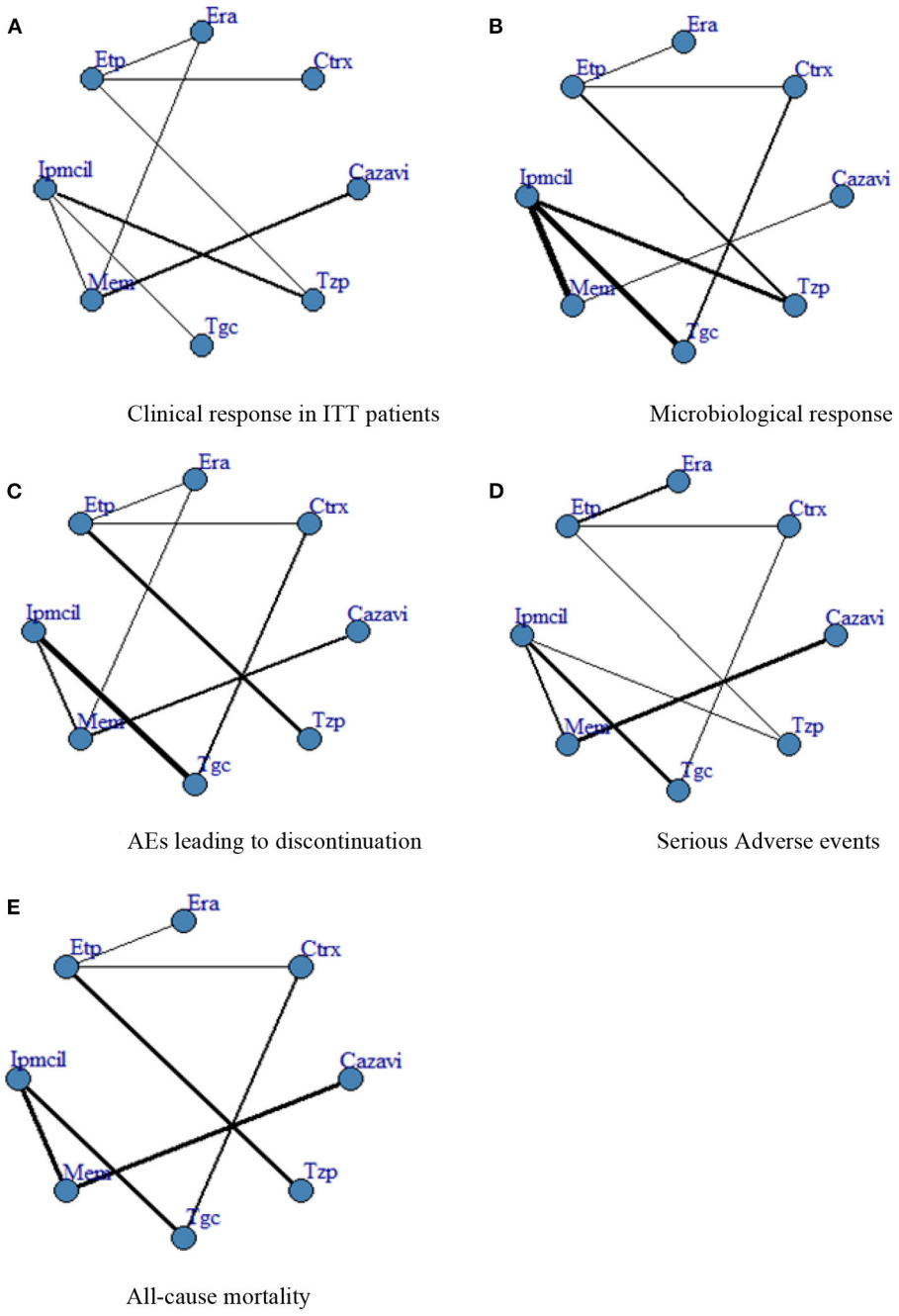

FIGURE 3
(A–E) Network plot. ITT, intention-to-treat; Era, eravacycline; Etp, ertapenem; Mem, meropenem; Tgc, tigecycline; Cazavi, ceftazidime/avibactam + metronidazole; Tzp, piperacillin/tazobactam; Ctrx, ceftriaxone + metronidazole; Ipmcil, imipenem/cilastatin.

0.91, 95%CI (0.58,1.40)], ceftazidime/avibactam plus metronidazole [Cazavi vs. Era: RR = 0.95, 95%CI (0.67,1.33)], imipenem/cilastatin [Ipmcil vs. Era: RR = 0.98, 95%CI (0.70,1.33)], meropenem [Mem vs. Era: RR = 1.00, 95%CI (0.76,1.30)] (Figure 4A). Meanwhile, no significant difference was observed between eravacycline and other comparators in CE patients (20 RCTs, 7,016 patients, Figure 4B) nor ME patients (15 RCTs, 3,756 patients, Figure 4C). The comparison outcomes of clinical response are presented in Supplementary Table 7.

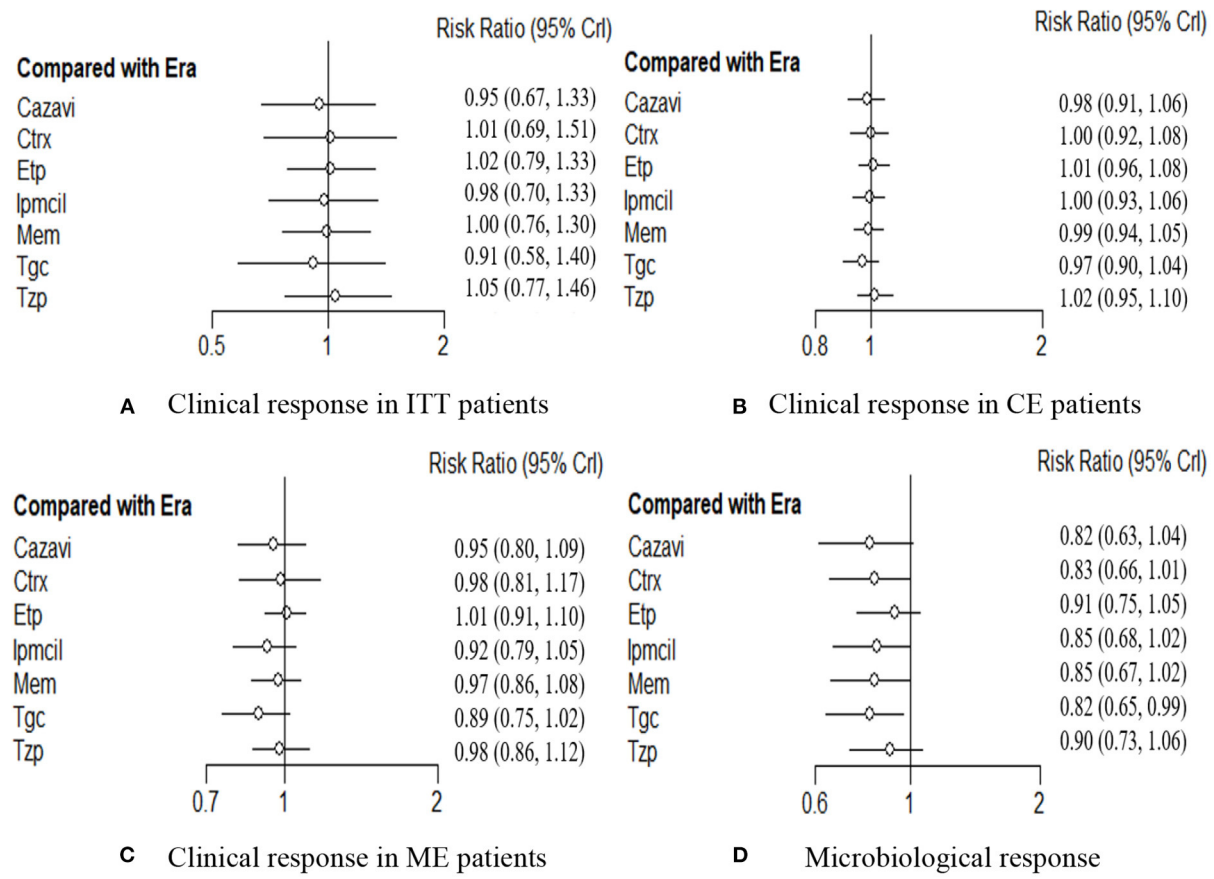

FIGURE 4
(A–D) Forest plot for efficacy endpoints. ITT, intention-to-treat; CE, clinical evaluate; ME, microbiological evaluable; Era, eravacycline; Etp, ertapenem; Mem, meropenem; Tgc, tigecycline; Cazavi, ceftazidime/avibactam + metronidazole; Tzp, piperacillin/tazobactam; Ctrx, ceftriaxone + metronidazole; Ipmcil, imipenem/cilastatin.

Network meta-analysis for microbiological response

Microbiological response was evaluated by 3,677 investigators in the 19 RCTs. Network meta-analysis showed significant efficacy of eravacycline compared with that of tigecycline [Tgc vs. Era: RR = 0.82, 95%CI (0.65,0.99)], but there was no significant difference from any of the other six comparators ($P > 0.05$, Figure 4D). The comparison outcomes of microbiological response are presented in Supplementary Table 8.

Network meta-analysis for safety

The rate of any AEs leading to discontinuation in 16 RCTs including 7,742 patients was evaluated. In addition to meropenem, eravacycline showed a lower adverse event discontinuation rate in treating cIAIs than the other six antibacterial drugs, but no significant difference was observed (Figure 5A). Additionally, differences did not reach statistical significance between eravacycline and the other drugs in the SAE rate (14 RCTs, 6,483 patients, Figure 5B) and all-cause mortality rate (16 RCTs, 7,766 patients, Figure 5C). The comparison outcomes of safety indicators are presented in Supplementary Table 9.

Publication bias

A funnel plot of clinical response for the ITT population was tested for publication bias and showed a generally symmetrical left-right distribution across each study point (Figure 6), which,

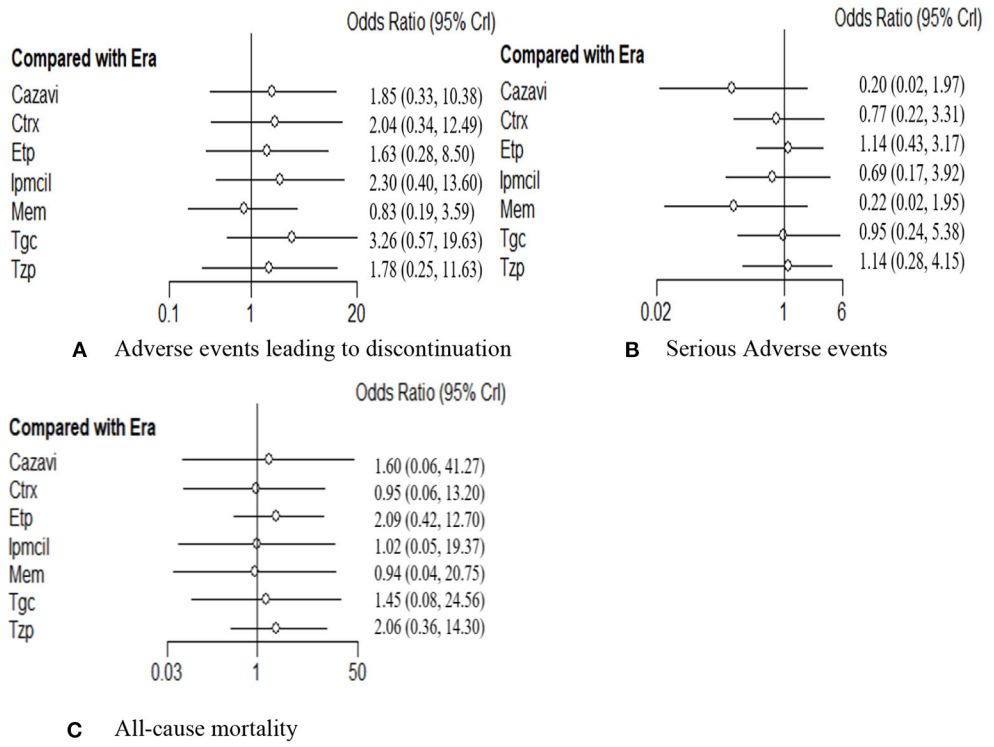

FIGURE 5
(A–C) Forest plot for safety endpoints. ITT, intention-to-treat; CE, clinical evaluate; ME, microbiological evaluable; Era, eravacycline; Etp, ertapenem; Mem, meropenem; Tgc, tigecycline; Cazavi, ceftazidime/avibactam + metronidazole; Tzp, piperacillin/tazobactam; Ctrx, ceftriaxone + metronidazole; Ipmcil, imipenem/cilastatin.

combined with the results of Begg's test ($p = 0.79$), suggested a low likelihood of publication bias.

Discussion

This study systematically searched RCTs of eravacycline and other clinically commonly used antimicrobial drugs in China for the treatment of adults with cIAIs and indirectly compared their efficacy and safety by Bayesian network meta-analysis. Based on the evidence generated from the current noninferiority clinical trial design, the clinical response rate of eravacycline for the treatment of adults with cIAIs was not statistically significantly different from that of the other seven clinically used antimicrobial drugs; the microbiological response rate was better than that of tigecycline, which was a statistically significant difference. In terms of safety, the incidence of serious adverse events, discontinuation rates, and all-cause mortality rates were also not statistically significantly different from the other seven treatment regimens for eravacycline.

Expert consensus on multidisciplinary management of intra-abdominal infections (1) recommends the selection of antimicrobial agents based on whether the patient is at high risk of treatment failure or death, the presence of serious infections, possible pathogens, the likelihood of multiple pathogenic infections, the local resistance rate of common causative organisms, and the risk of infection with drug-resistant organisms. Cephalosporins, carbapenems and enzyme inhibitor combinations are the first-line mainstream agents for empirical anti-infective therapy in hospital-acquired and high-risk community-acquired intra-abdominal infections, while ceftazidime/avibactam, tigecycline and eravacycline are used as appropriate therapies for target treatment. In clinical practice, drug selection remains difficult due to the lack of evidence of direct comparisons between different carbapenems, newer tetracyclines (tigecycline, eravacycline) and other antibacterial drugs.

In terms of efficacy, this study demonstrated no statistically significant difference between the clinical response rate of eravacycline and ceftriaxone, carbapenems and other commonly used antimicrobials. Lan (38) and Eljaaly (39) conducted meta-analyses of the efficacy and safety of eravacycline vs. meropenem and ertapenem for the treatment of cIAIs in 2019 and 2021, respectively, and their results showed that there was no statistically significant difference between the clinical response rate of eravacycline and the two carbapenem

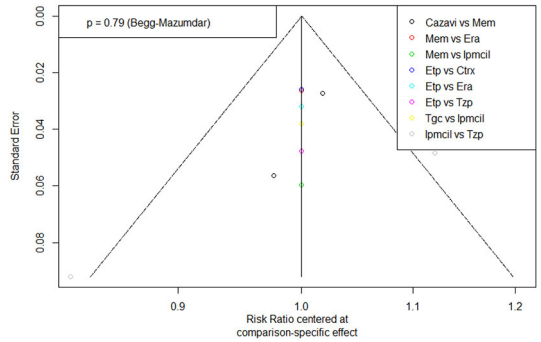

FIGURE 6
Funnel plot of Publication bias. Era, eravacycline; Etp, ertapenem; Mem, meropenem; Tgc, tigecycline; Cazavi, ceftazidime/avibactam + metronidazole; Tzp, piperacillin/tazobactam; Ctrx, ceftriaxone + metronidazole; Ipmcil, imipenem/cilastatin.

antimicrobials, which is consistent with the results of this study. However, in real-world clinical applications in China, resistance to cIAIs is serious, as the common pathogens of cIAIs are predominantly gram-negative bacilli. Taking ESBL enterobacteria commonly found in complicated abdominal infections as an example, the susceptibility of ESBL *Escherichia coli* infections to ceftriaxone was <1% in both cases, and the susceptibility of ESBL-producing *Klebsiella pneumoniae* to ceftriaxone and cefotaxime was only 1.7% (40). The emergence and prevalence of carbapenem-resistant strains has been caused by the high level of resistance of Enterobacteriaceae bacteria to the three generations of cephalosporins, further increasing the use of carbapenems. In China, the resistance rate of *Klebsiella pneumoniae* to carbapenem-resistant antimicrobials has rapidly increased from 3% in 2005 to more than 25% in 2021 and even up to more than 35% in some provinces (41). The results of a retrospective observational study conducted by Cancelli (42) showed that carbapenem-resistant patients treated with carbapenems had a lower clinical cure rate than nonresistant patients (resistant vs. nonresistant patients: 78.2 vs. 91.8%, $p = 0.03$). Thus, cephalosporins and carbapenems have poor clinical efficacy and high failure rates in the treatment of cIAIs, particularly in patients with resistant bacterial infections. Solomkin et al. [9, 12] demonstrated that the clinical cure rate of eravacycline in patients with ESBLs-producing pathogens was 100%, and in patients with ESBLs-producing Enterobacteriaceae was as high as 87.5%, with superior efficacy.

Eravacycline and tigecycline are new tetracycline antibacterial agents. The results of comparison of eravacycline with tigecycline show that in terms of efficacy, eravacycline has a higher numerical clinical response rate and a significantly better microbiological response rate than tigecycline. These results can be partly explained by the better antibacterial activity of eravacycline. Eravacycline optimizes its antibacterial activity through a unique modification of the core D-ring of tetracycline. Eravacycline has a 2- to 4-fold lower minimum inhibitory concentration MIC_{90} than tigecycline against common gram-negative bacteria in both the overall and multidrug-resistant populations (43, 44) and has better *in vitro* antibacterial activity. In terms of safety, the absolute values of AE discontinuation rates were lower for eravacycline than for tigecycline. Furthermore, the results of a pooled analysis of tigecycline all-cause mortality in clinical studies by McGovern (45) and Prasad (46) suggested that tigecycline may increase the risk of death. Therefore, compared to tigecycline, eravacycline has better efficacy in absolute value and better safety.

The key finding of this study is supported by the following evidence. First, the 25 RCTs included were of high quality and had a low risk of bias; thus, the results of network meta-analysis were highly credible and convincing. Second, the study was more comprehensive in its consideration of outcome indicators, considering the clinical response rates of the ITT, CE, and ME populations in terms of efficacy indicators and the rates of adverse discontinuation, mortality, and serious adverse events in terms of safety, making the conclusions more reliable and stable. Again, the drugs included in this study for the treatment of cIAIs were comprehensive, comparing the clinical efficacy and safety of the new antimicrobial drug eravacycline for the treatment of cIAIs with seven commonly used antimicrobial drugs in China. These results provide a reference for clinicians in terms of clinical decision-making and antimicrobial drug application.

This meta-analysis has certain limitations. First, as the definitions of clinical response rates and microbiological response rates for patients with cIAIs were not entirely consistent across RCTs, which may have led to some bias in the results of the network meta-analysis. Second, considering the lack of high-quality cohort studies related to cIAIs antibacterial drugs in China, we can only make recommendations and references based on published RCTs worldwide. Therefore, the results need to be verified by further RCT studies in Chinese patients. Third, due to the absence of patient's resistance data in the original studies included, we were unable to perform a meta-analysis of the efficacy for different baseline pathogens. but published studies have demonstrated the severity of cephalosporin- and carbapenem-resistant strains, and the superior efficacy of eravacycline against extended-spectrum beta-lactamase (ESBL)-producing pathogens has also been confirmed [9, 12, 40–42]. Fourth, considering the increasing antimicrobial resistance, the efficacy of the antibiotics might change throughout years, which introduces some uncertainty to the results. Finally, due to limited data acquisition, the baseline characteristics of patients could not be matched to be completely consistent, and there were only three studies related to eravacycline in the included studies. Further direct comparative studies are needed to confirm the results.

Conclusions

Based on the evidence generated by the current noninferiority clinical trial design, the efficacy and safety of eravacycline for the treatment of cIAIs in adults was not statistically significantly different from the other seven clinically commonly used antimicrobial drugs. In terms of microbiological response rates, eravacycline was statistically significantly better than tigecycline. In view of the severe resistance situation of penicillin, cephalosporins and carbapenems in China and the difficulty of existing drugs to meet clinical treatment needs, the new antimicrobial drug eravacycline may be one of the preferred options for the treatment of cIAIs in adults.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding authors.

Author contributions

RM, XG, AM, and HL: conception of the study. RM, LS, and ZF: literature search and data extraction. RM, XG, LS, and ZF: statistical analysis. RM, YL, and ML: checked the data and drafting the manuscript. AM and HL: revising and completion of final work. All authors reviewed and approved the final version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.935343/full#supplementary-material

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Antiviral properties of porous graphene, graphene oxide and graphene foam ultrafine fibers against Phi6 bacteriophage

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As the world has experienced in the Coronavirus Disease 2019 pandemic, viral infections have devastating effects on public health. Personal protective equipment with high antiviral features has become popular among healthcare staff, researchers, immunocompromised people and more to minimize this effect. Graphene and its derivatives have been included in many antimicrobial studies due to their exceptional physicochemical properties. However, scientific studies on antiviral graphene are much more limited than antibacterial and antifungal studies. The aim of this study was to produce nanocomposite fibers with high antiviral properties that can be used for personal protective equipment and biomedical devices. In this work, 10 wt% polycaprolactone-based fibers were prepared with different concentrations (0.1, 0.5, 1, 2, 4 w/w%) of porous graphene, graphene oxide and graphene foam in acetone by using electrospinning. SEM, FTIR and XRD characterizations were applied to understand the structure of fibers and the presence of materials. According to SEM results, the mean diameters of the porous graphene, graphene oxide and graphene foam nanofibers formed were around 390, 470, and 520 nm, respectively. FTIR and XRD characterization results for 2 w/w% concentration nanofibers demonstrated the presence of graphene oxide, porous graphene and graphene foam nanomaterials in the fiber. The antiviral properties of the formed fibers were tested against *Pseudomonas phage* Phi6. According to the results, concentration-dependent antiviral activity was observed, and the strongest viral inhibition graphene oxide-loaded nanofibers were 33.08 ± 1.21% at the end of 24 h.

KEYWORDS

graphene oxide, porous graphene, graphene foam, antiviral, nanofiber, electrospinning, Phi6 bacteriophage

Introduction

Viruses are nanosized obligate intracellular parasites that need a living host cell to survive and reproduce (1). They cause viral infections which can result in a significant level of morbidity and mortality, like the current Coronavirus Disease 2019 (COVID-19) pandemic threatening the world (2, 3). There are a number of ways to treat viral infections, such as developing vaccines and antiviral drugs, however, some viruses can overcome these treatments because they mutate rapidly (4). Thus, people are primarily encouraged to prevent contamination by using personal protective equipment (PPE) (5–7). Transmissibility of a virus depends on the virus variation, and the routes of transmission might be divided into direct (person-to-person) contact, indirect (object-to-individual) contact, droplets and aerosols (8). Adenovirus, enterovirus, metapneumovirus, rhinovirus (RV), influenza, respiratory syncytial virus (RSV) and coronavirus are among the pathogens that cause respiratory tract infections (9). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the cause of COVID-19 emerged in China and spread rapidly around the world, consequently affecting millions of people (3). In this challenging time, face masks, hand sanitizing and social distancing have been recommended by the World Health Organization (WHO) and governments as the first step in a comprehensive prevention strategy to suppress COVID-19 transmission and save lives (10). It has been reported by researchers that SARS-CoV-2 can maintain its viability in aerosols for up to 3 h (11, 12). Microdroplets emitted into the air when coughing, talking, or breathing are the main sources of airborne transmission of these viruses (13). Bacteriophage Phi6 belongs to the *Cystoviridae* family and infects *Pseudomonas* bacteria. Similar to SARS-CoV-2 it is enveloped by a lipid membrane, has spike proteins, and is of similar size (80–100 nm), thus making it a good surrogate for studying RNA viruses (14, 15).

Graphene is a two-dimensional (2D) honeycomb shape lattice of carbon atoms that was initially prepared by micromechanical cleavage of bulk graphite (16, 17). Graphene has emerged as one of the most promising nanomaterials that have attracted the scientific community's interest because of its unique combination of extraordinary properties such as high electrical and thermal conductivity, high purity, good bio-functionalizability, solubility, the capability of easy cell membrane penetration for high antimicrobial effects, high surface area and theoretical strength (18–22). These unusual properties of graphene offer a fascinating material platform in biomedical research like wearable electronics (23), ultrasensitive biosensors (24, 25), tissue engineering (26, 27) and antimicrobial filtration (18–20). Polymer composite fibers containing antimicrobial agents (graphene derivatives, copper (Cu), curcumin, chitosan, tellurium, titanium dioxide (TiO_2), etc.) play a crucial role in the development of PPE, wound dressing films and filters to reduce the level of microbial contamination and bioburden (28–32).

The antimicrobial activity of graphene and its derivatives is due to a combination of physicochemical properties. These can be listed as oxidative-stress mediated, layer number, lateral size effect, tailored surface, time and concentration dependency (33–36). Oxidative stress mediated antimicrobial activity containing functional groups (hydroxyl, epoxy, carboxyl) is generally seen in graphene oxide (GO) and reduced graphene oxide (rGO), as functional groups increase, the antimicrobial effect also increases (37). Since graphene has sharp edges, it is expected that the antimicrobial activity will decrease as the lateral size increases (38). The antimicrobial effect will also increase as the layer number increases (38). Additionally, agglomeration of graphene nanomaterials gives rise to antimicrobial activity, as it reduces microbial interaction and prevents nutrients from reaching the microbes (39). The increase in the exposure time and the dose of the material also increases the antimicrobial activity.

Many studies have been reported on graphene and its derivatives and their nanocomposites discussing antibacterial, antifungal and antiviral activities (18–20, 40–42). Matharu et al. investigated the antibacterial activity of 2, 4 and 8 w/w% concentration graphene nanoplatelet (GNP) and GO loaded polymethylmethacrylate (PMMA) composite nanofibers against *Escherichia coli (E. coli)* (19, 20). According to these dose-dependent results, GO and GNP loaded with 8 w/w% showed the highest antimicrobial efficiency, around 85% and 95% respectively. Likewise, in another study, the antibacterial properties of GO added to polyurethane (PU) polymer was observed at different concentrations (0, 1, 5, 10 wt%) (43). It was concluded that *E. coli* and *Staphylococcus aureus (S. aureus)* bacteria decreased the at most at the highest concentration level (10 wt%). Since this study was conducted for water purification application, it is not a concern of toxic effects on the human body, but it is essential for graphene-based materials for biomedical research.

GO, porous graphene (PG) and graphene-based foam (GF) are among the graphene derivatives (**Figure 1**) sought after. PG refers to graphene-related materials that have nano-sized pores on the basal plane, the size and distribution of which differ according to the synthesis method. PG, which has structural properties very close to pristine graphene, can be synthesized by chemical and physical means (44). It is used especially for gas separation and purification applications paying attention to its high permeability (44, 45). PG polymer composite nanofibers have been formed using pressurized gyration and the surface topography was studied by Ahmed et al. (40). According to this study, PG was seen as a promising material in ultrafiltration applications (40). GO is a material that many researchers have examined, especially its antibacterial activity as mentioned above (20, 46, 47). GO contains functional groups causing high levels of oxidative

stress that play a major role in its antimicrobial effect against pathogens (33). Matharu et al. reported the antiviral activity of GO, here the mechanism could be a physical and chemical interaction and GO virucidal action increases depending on time and concentration (18). GF is a graphene derivative with a high surface area providing a uniform and homogeneous distribution of graphene in biomedical applications (48, 49). Unlike PG and GO, GF has a 3-dimensional structure and it has low density. Wang et al. reported that GF shows significant biomineralization in engineering, scaffolds formed from the GF-polycaprolactone (PCL) composite are a good example (49). No significant cytotoxicity was found in liver and kidney macrophages for 7 days, according to GF biocompatibility and toxicity assessments (48).

In this study, the antiviral activity of electrospun PG, GO and GF nanocomposite fibers were assessed against a double-stranded RNA virus. Electrospinning is one of the most common fiber production methods used to obtain nano-sized polymeric fibers from various polymer solutions applying high voltage. PCL was used as the carrier polymer as it is a biocompatible, easily processable polymer, and when it is dissolved in acetone, a non-toxic environmentally friendly solution is obtained when used in the human body. Previous antimicrobial studies of graphene were mostly focused on the antibacterial and viral inhibition was not included in much research. At the same time, the antiviral activity of PG and GF are investigated for the first time in this study. These comparative antiviral fibrous structures enable us to find the optimum material for PPE for future antiviral filtration systems.

Materials and methods

Materials and preparation of solutions

PCL (Mw ∼ 80,000 g/mol), GO (average number of layers 15–20) and acetone were purchased from Sigma Aldrich (Gillingham, UK). PG (pore size around 3–5 nm) and GF (sized about 4 μm with folded area and number of layers varied from 2 – 3 to 9 – 15) were synthesized as reported by Tabish et al. in the previous research (40, 48, 50).

PG, GO, GF powders and PCL polymer (10 wt%) were weighted on precision scales for 5 different concentrations (0.1, 0.5, 1, 2, 4 w/w%) determined as indicated in **Table 1**, and were suspended into acetone solvent. The solutions were prepared in two separate parts and mixed at the end. The first part is PCL and acetone solution, and the second part is nanomaterial and acetone suspension. PG, GO and GF nanomaterials were calculated as in the table and added to the acetone solvent. The PG, GO and GF suspensions were then sonicated in an ice bath to achieve a homogenized solution with a Branson SFX 550 Digital Probe Sonifier (Cole-Parmer, Eaton Socon, UK) set at 80–100% for approximately 2 h and then left overnight on a magnetic stirrer (**Figure 2**). In order for the PCL pellets to dissolve in acetone, they were left overnight on a heated magnetic stirrer set at 50°C. After the polymers were completely dissolved, they were mixed with the homogenized suspension solutions after ultrasonication was completed. Then all solutions were left on the magnetic stirrer for ∼4 h.

Surface tension and viscosity

Surface tension measurement of 15 different nanocomposite solutions was performed using Kruss Tensiometer (Tensiometer K9, Hamburg, Germany). During this measurement using the Du Nouy ring method, approximately 10 mL of solution was taken from the glass bottle and a platinum-iridium ring was dipped into it and slowly withdrawn. In order to ensure correct results, the ring was first calibrated with distilled water and then used for solutions. The maximum surface tensions obtained during extraction were recorded. This process was performed three times for each solution and the averages were recorded. The ambient temperature was recorded as approximately 23°C.

A Brookfield Viscometer DV-III was used to determine the viscosity of the solutions (Brookfield, Middleboro, MA,

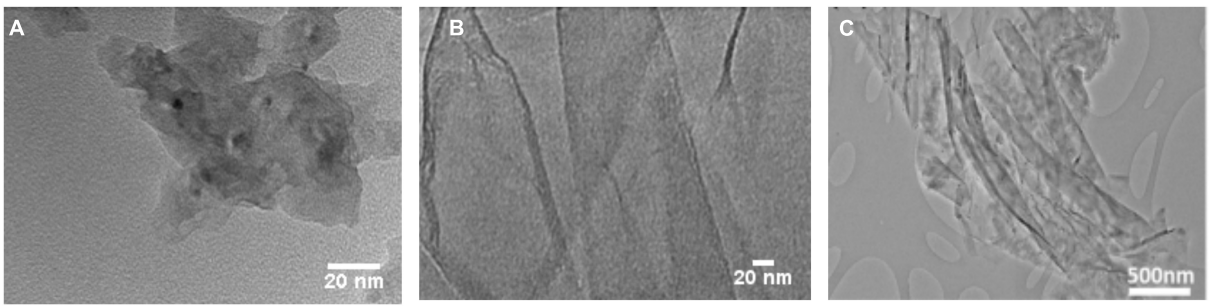

FIGURE 1
High-resolution transmission electron microscopy characterization of **(A)** PG **(B)** GO **(C)** GF. In **(C)** has been reproduced with permission from reference (48) Copyright 2017, Materials.

TABLE 1 Composition of porous graphene, graphene oxide and graphene foam loaded polycaprolactone (PCL) nanofiber solutions.

Final concentration (w/w%)	PCL (g)	Solvent (acetone) (mL)	PG (g)	Solvent (acetone) (mL)	GO (g)	Solvent (acetone) (mL)	GF (g)	Solvent (acetone) (mL)
0	5	50	–	–	–	–	–	–
0.1	5	25	0.005	25	0.005	25	0.005	25
0.5	5	25	0.025	25	0.025	25	0.025	25
1	5	25	0.05	25	0.05	25	0.05	25
2	5	25	0.1	25	0.1	25	0.1	25
4	5	25	0.2	25	0.2	25	0.2	25

USA). For each of the solutions, approximately 6 ml was poured into the viscometer and the values measured with a small-sample spinner were recorded. Viscosity measurements of nanocomposite solutions were carried out at ambient conditions (23°C) and were performed three times to get an average result.

Fabrication and characterization of loaded fibers

PG, GO, and GF incorporated fibers were collected using a grounded metal flat plate collector (Figure 2). The needle used was 18G (1.25 mm BD micro lance) and the capillary tube was polytetrafluoroethylene (PTFE) (outer diameter 2 mm and inner diameter 1 mm). DC power supply provides an applied voltage of 12–16 kV. The distance between the needle tip and the collector was 150 mm, and the flow rate was set at 0.2 mL/min. The humidity in the room during electrospinning was recorded in the range of 45–53%, the temperature was measured in the range of 23–26°C.

The fibers were gathered after manufacture, mounted on aluminum studs, and gold sputter coated for 90 s (Q150R ES Quorum Technologies Ltd., Laughton, UK). After that, these samples were examined using a scanning electron microscope (SEM) (Hitachi S-3400n, Tokyo, Japan) with a 5 kV working voltage. The morphology of the nanocomposite fiber mats were determined using SEM at about 10 different areas of a sample. A total of 100 fibers were randomly measured using Image J software, and the mean diameter and standard deviation were computed using Excel. OriginPro software was used to create the histogram graphs of the frequency distribution of fiber diameters.

Fourier transform infrared spectroscopy (FTIR) (Bruker Optics Tensor-27 IR, Ettlingen, Germany) characterization with a wavelength range of 4,000–500 cm^{-1} was applied to pure PCL and 2w/w% PG-PCL, GO-PCL, GF-PCL nanofibers. Nanofiber samples were adjusted to be approximately 2 mm thick and 5 mm in diameter and placed in the spectrometer. X-ray diffraction (XRD) (at 40 kV and –40 mA) characterization was used for 4 different samples at the same concentration.

Antiviral studies

In this work, *Pseudomonas* Phi6 bacteriophage was used to model double-stranded RNA viruses. *Pseudomonas syringae* (*P. syringae)* and *Pseudomonas* Phi6 bacteriophage were sourced from DSMZ (Braunschweig, Germany). The received microorganisms were cultured following the manufacturer's instructions. Stock cultures of *P. syringae* were stored in a Microbank^{TM} at −20°C, whilst the Phi6 bacteriophage was stored in a cool dark place. Antiviral activity was assessed against this microorganism as it is a safe, easy to work with and well-studied model surrogate for SARS-CoV-2.

Actively growing broth cultures of *P. syringae* were prepared by incubating a single colony in 30 ml of tryptone soya broth for 24 h at 25°C and 150 rpm.

Bacteriophage suspensions containing the made fibers in PBS were prepared. A total of 100 μl of the suspension at 0 and 24 h was added to 300 μl of the overnight *P. syringae* culture and 3 ml of molten semi-solid agar (0.5% agar) and poured onto agar plates. The plates were incubated for 24 h at 37°C and the number of plaques was counted. The viral reduction was calculated by comparing the number of virions at 24–0 h. Antiviral activity was statistically analyzed and compared to the control samples using unpaired *t*-tests. The difference was considered significant when $p < 0.05$.

Results and discussion

Suspension behavior

Graphene suspensions were prepared in different concentrations (0.1, 0.5, 1, 2, 4 w/w%) as PCL-PG, PCL-GO and PCL-GF. The fibers obtained from these suspensions were formed by the electrospinning method, which is affected by the surface tension and viscosity values of the solution.

In Figure 3A, the surface tension values of the pure PCL solution and nanocomposite solutions loaded with PG, GO and GF at increasing concentrations are shown. According to the results, the pure PCL solution has relatively higher

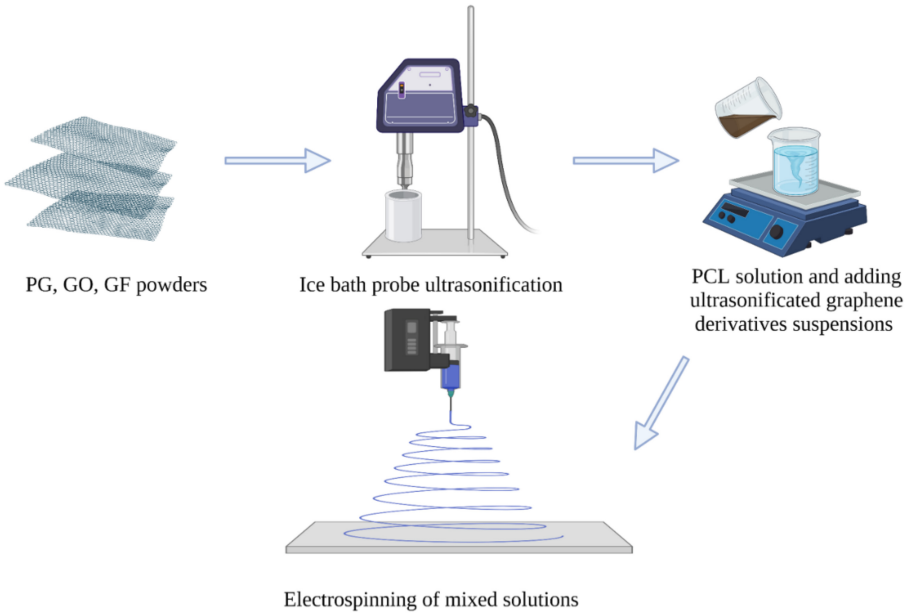

FIGURE 2
Schematic demonstrating the production of PG, GO and GF-based polymer composite fibers with electrospinning. All images within this figure are prepared in Biorender.com and have been used with permission from Biorender.

surface tension than the fluids with PG-GO-GF added. The range of decrease was observed as approximately ∼1.3% for PCL-PG, ∼3.9% for PCL-GO and ∼3.4% for PCL-GF at different concentrations. The pure PCL solution had an average surface tension of 25.6 ± 0.7 mN/m, this decreased to around 25.3 ± 0.4 mN/m upon the addition of 0.1 w/w% PG, however, this value did not change much with the increase of the concentration and showed a stable behavior. Likewise, the surface tension of 0.1 w/w% GO and GF dropped to 23.7 ± 0.3 and 22.1 ± 0.4 mN/m respectively, and then near-constant values were observed with increasing concentrations. According to the results in this study and literature, the addition of surfactants result in a reduction in surface tension (51). Similarly, graphene-based materials act as a surfactant and the electric force between the particles causes surface tension reduction in this study. Surface tension has a counteracting effect, and a lower voltage is required for jet initiation when surface tension is reduced. Surface tension also has a direct impact on the generation of beads (51).

The viscosity effect of graphene materials added to the PCL solution is shown in **Figure 3B**. Initially, the viscosity of the pure PCL solution was approximately 49.84 ± 0.2 mPa.S and a moderate decrease was observed with the addition of graphene derivatives. While the viscosity values of the suspensions with PG added were 23.99 mPa.s on average, this value slightly decreases to 23.07 and 23.2 mPa.S for GO and GF, respectively. The approximate range results was 4.58 mPa.S of PCL-PG solution, 1.92 mPa.S for PCL-GO, and 3.1 mPa.S for PCL-GF at different concentrations. The decrease in viscosity may be caused by the instability of the added nanomaterial in the solution, due to the heat of the applied solvent and polymer.

Fiber characterization

0.1, 0.5, 1, 2 and 4 w/w% PCL-PG, PCL-GO and PCL-GF nanofibers were prepared using electrospinning. The prepared fibers were imaged using SEM to understand fiber morphology. A histogram was created with fiber diameter distribution by measuring the diameter of 100 sample fibers. The formation of some large beads was observed due to the rapid evaporation of the solvent acetone used during the electrospinning process. Due to the nature of the electrospinning method, the fibers dispersed with the aid of high voltage are not uniformly aligned. However, it is observed that adding graphene nanoparticles, which are affected by high voltage with increasing concentrations, increases the entanglement of fibers. While the applied high voltage is necessary to overcome the surface tension, the electrical conductivity of the nanomaterial used is affected by this high voltage, causing asymmetric fiber formation (52).

As seen in **Figure 4**, PCL-PG nanofibers were tubular with some beaded, porous surface properties. The mean fiber diameter of 0.1 w/w% PG-loaded PCL was found to be approximately 390 ± 170 nm. At the highest concentration 4 w/w%, a slight diameter increase of 420 ± 181 nm was observed. Although the uniform distribution of fibers was obtained in PCL-PG polymer nanocomposite fibers in general,

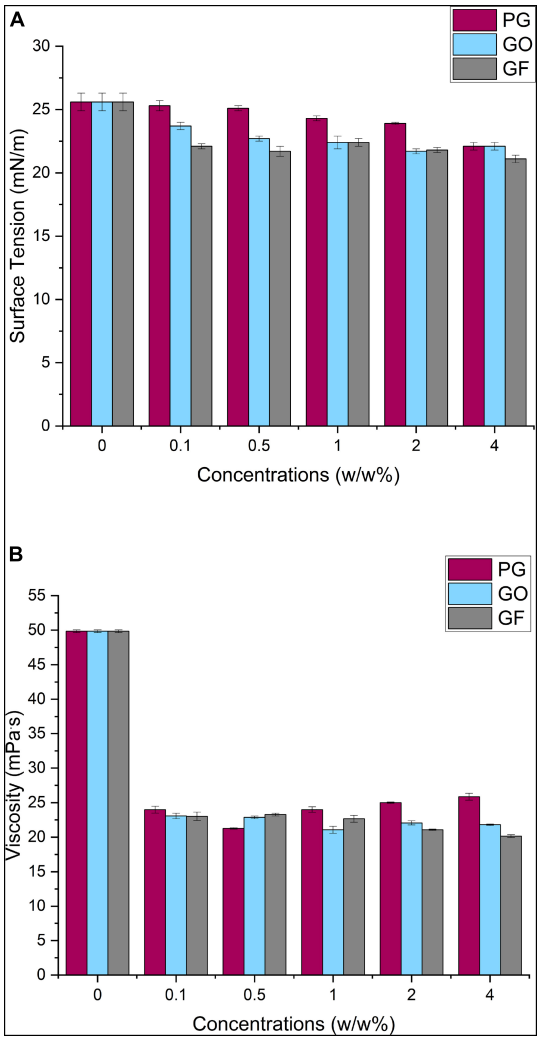

FIGURE 3
PCL-PG, PCL-GO and PCL-GF solutions surface tension **(A)** and viscosity **(B)**.

the thickest fiber is calculated at around 1.6 μm at 0.1 w/w% PCL-PG SEM images. While the fiber diameter for 0.1 w/w% PCL-PG was between 200 and 400 nm, it was observed that the uniformity of the other samples reduced.

SEM characterization of PCL-GO nanofibers and fiber diameter distribution is shown in Figure 5. Especially at high concentrations of PCL-GO fibrous, some bead formation is observed in SEM images. Bead formation might be related to the agglomeration of GO nanoparticles. While the dispersion of nanosized fibers showed linear alignment for 0.1 w/w% PCL-GO, GO nanoparticles affected by high voltage with increasing concentration aids curl-up of the fibers. Between the lowest and highest concentrations of GO nanofibers, the average fiber diameter was not very different, with an average of 460 ± 184 nm at 0.1 w/w% concentration, 530 ± 208 nm at 4 w/w% concentration, and no linear increase was found with increasing concentration. In general, a few thicker fibers appear above 1 μm in each GO sample diameter calculation.

The PCL-GF nanofibers shown in Figure 6 have a more porous and non-uniform structure than PG and GO. While the nanofiber diameter distribution of 0.1 w/w%, 0.5 w/w%, and 1 w/w% was almost the same, it is around 480 nm, and the mean fiber diameter moderately increased with an increasing concentration to approximately 660 ± 315 nm. Another difference in PCL-GF fibers was the standard deviations increasing with concentration and therefore decreasing uniformity. Fibers with a thickness greater than 2 μm were seen in fibers containing high concentrations of GF. The average diameters of the PG, GO and GF fibers were approximately 390, 470, and 520 nm, respectively. In summary, a mostly increase in the diameters of PG, GO and GF added nanofibers was observed depending on the concentration. This result supports that the added nanofiller increases its diameter by embedding in the fiber and/or adhering to the surface.

2 w/w% PG, GO, and GF nanofibers were analyzed by FTIR and XRD characterizations. FTIR analysis is performed to validate the presence of graphene derivatives in nanofibers. FTIR analysis of PG (40, 45), GO (45, 50, 53) and GF (48, 54) nanomaterials were performed in previous studies. FTIR peaks of pure PCL nanofiber were noticed at 2920, 1720, and 730 cm^{-1} (Figure 7). These peaks also existed in the case of other PCL-based nanofibers. The 1043 and 1720 cm^{-1} peaks encountered in the PG-PCL nanofiber correspond to the epoxy and carbon functional groups. These peaks and their corresponding functional groups are in consistent with previously published work (40). Similarly, GO-PCL nanofiber FTIR analysis generated peaks of carboxyl groups at 1,382 and 1030 cm^{-1}, as shown in previous studies (45, 53). Finally, the peaks at 1622 and 1386 cm^{-1} appeared in the case of GF-PCL nanofiber typically correspond to the presence of GF (48, 54). In summary, FTIR analysis results proved the existence of PG, GO and GF nanomaterials on sample nanofibers.

XRD results of pure PCL and graphene-PCL nanofibers are shown in Figure 8. Two distinct peaks are clearly seen in each graph. Since the XRD analysis results of pure PCL and other nanomaterials were similar, no significant shift in peaks was observed indicating the presence of PG, GO, and GF. These results can be interpreted as the fiber crystallization process is similar.

Antiviral activities

The viricidal properties of the nanocomposite fibers were tested against bacteriophage Phi6. A plaque assay was used to quantify the number of infectious viral particles in suspension before and after treatment. The advantage of using plaque assays is their ability to give a direct quantitative measurement of the exact number of virions in suspension (55, 56).

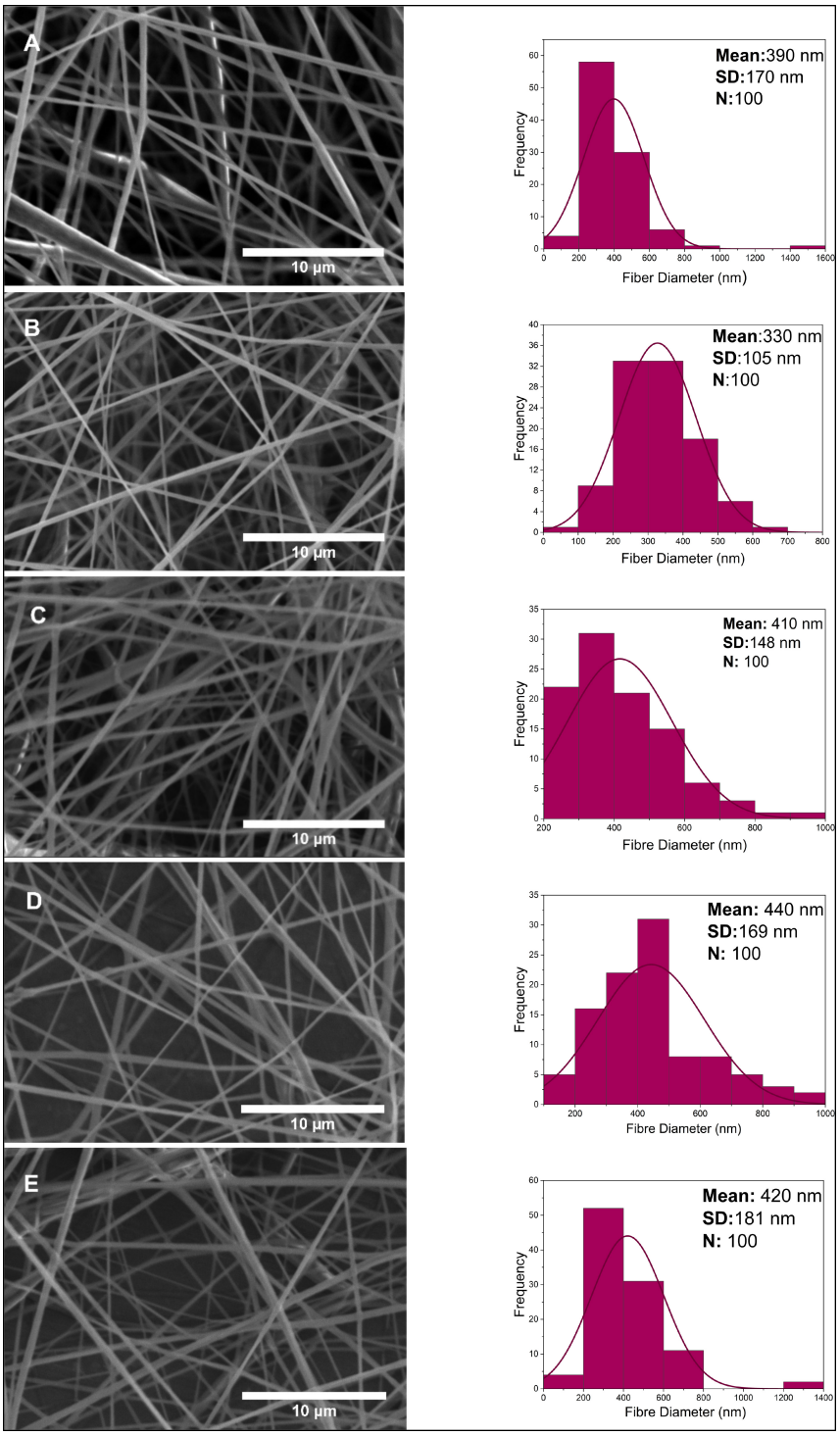

FIGURE 4
Scanning electron microscope images and fiber diameter distribution of nanofibers at 0.1, 0.5, 1, 2, 4 w/w% concentration for PG **(A–E)**.

As shown in Figure 9, pure PCL fibers (loaded with 0.0 w/w%) showed a slight reduction in virions (13.08 ± 1.38%). This is likely owed to the lack of host cells in the PBS to allow for viral survival and proliferation.

As seen in Figure 9, all nanocomposite fibers showed antiviral activity at the highest concentration tested. After 24 h of exposure, PCL fibers containing 4.0 w/w% of GO nanoparticles showed the strongest antiviral activity, with an average viral

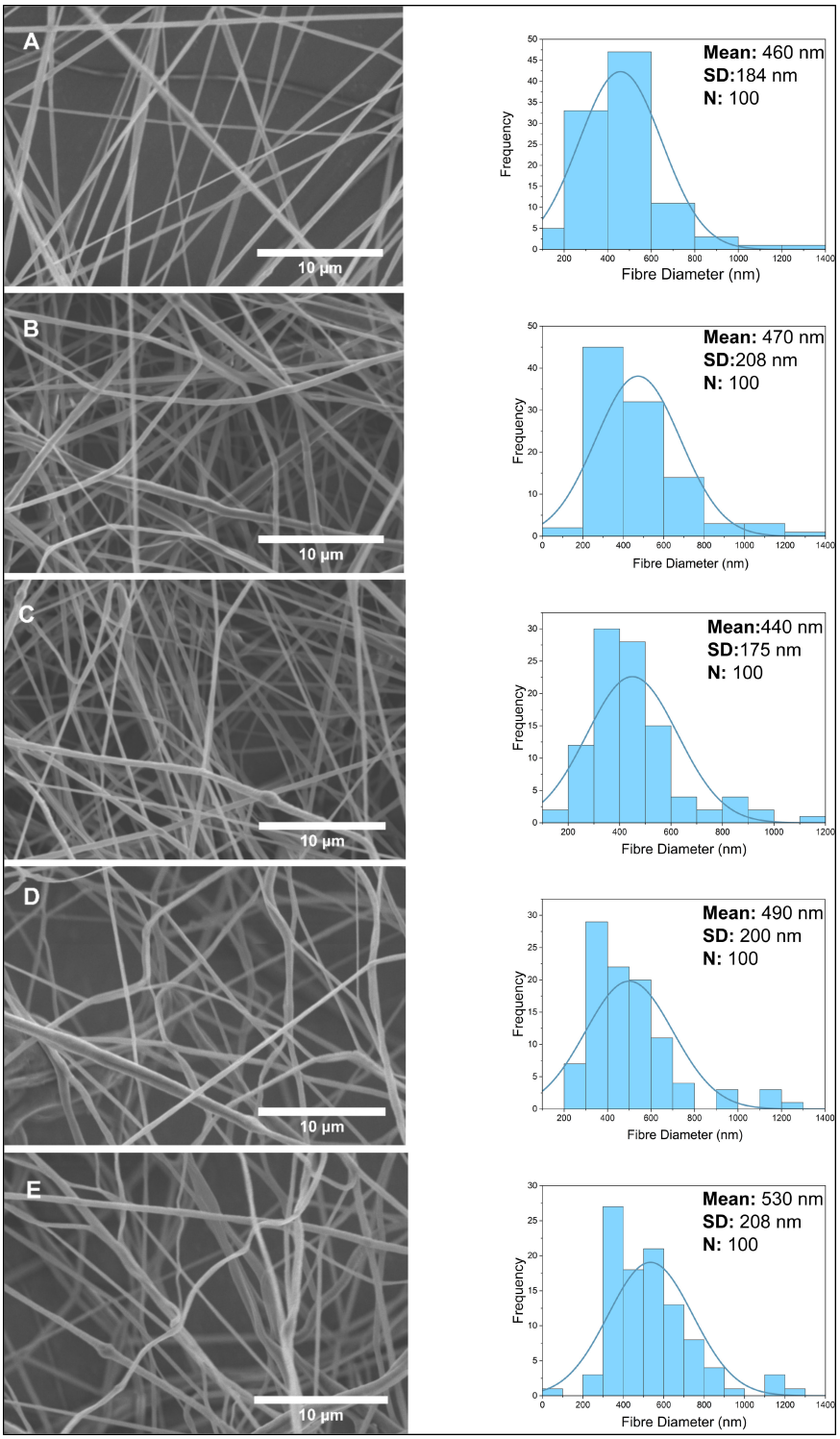

FIGURE 5
Scanning electron microscope images and *fiber* diameter distribution of nanofibers at 0.1, 0.5, 1, 2, 4 w/w% concentration for GO **(A–E)**.

reduction of 33.08 ± 1.21% (p-value = 0.0008). PCL fibers loaded with GF and PG at the same concentration, showed similar but slightly lower antiviral activity, with reductions of 31.2 ± 1.11 and 30.5 ± 2.0%, respectively. Fibers containing the lowest nanoparticle concentration (0.1 w/w%) exhibited viral reductions of 13.4 ± 1.9, 15.4 ± 4.9, and 14.01 ± 1.8%, for GO,

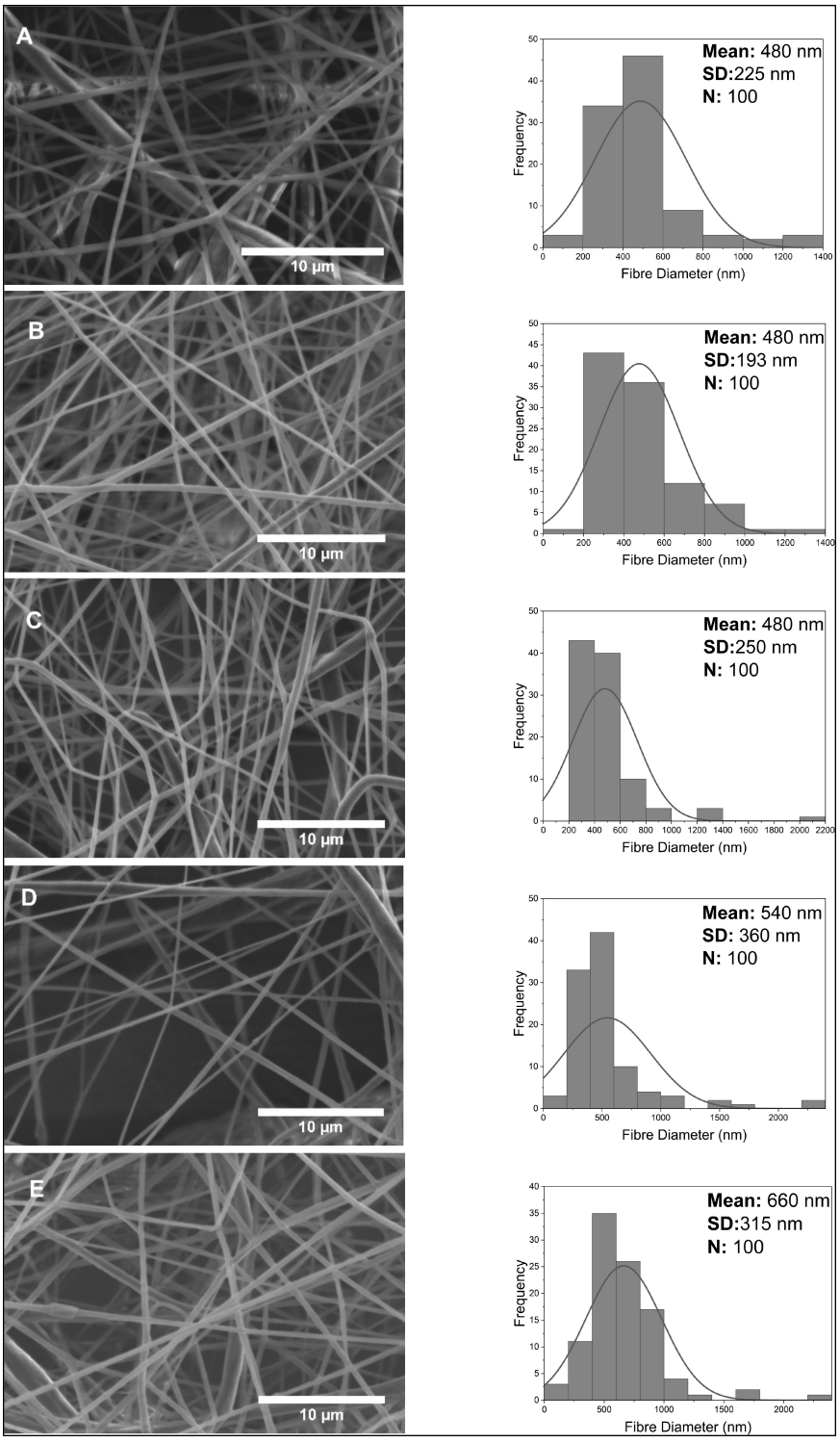

FIGURE 6
Scanning electron microscope images and *fiber* diameter distribution of nanofibers at 0.1, 0.5, 1, 2, 4 w/w% concentration for GF **(A–E)**.

GF and PG, respectively (the difference between the pure fibers and loaded fibers is not statistically significant for all materials at this concentration). At a concentration of 0.5 w/w% of GF or PG, fibers showed a statistically significant viral reduction when compared to the control. Whereas GO fibers only showed a statistically significant reduction at a concentration

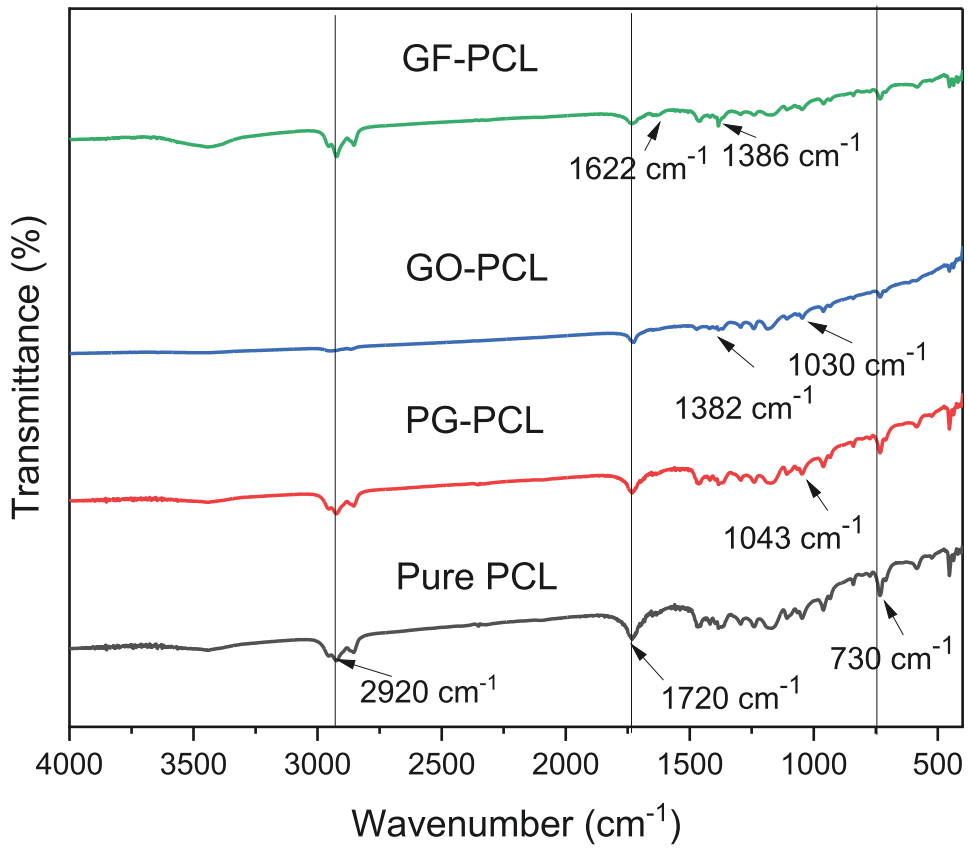

FIGURE 7
Fourier transform infrared spectroscopy analysis of pure PCL and 2w/w% PG-PCL, GO-PCL, GF-PCL nanofibers.

of 1.0 w/w% or more. This indicates that porous-like structures are more effective at lower concentrations. Whereas at higher concentrations, solid structures are more effective, likely due to their increased exposed surface area.

Overall, the results shown in **Figure 9** show the antiviral activity of all materials tested to be concentration-dependent. Increasing the loading increases the concentration of graphene-based nanomaterials on the fiber surface, therefore increasing the area of exposed material.

Discussion

It has been explained in previous studies that graphene and its derivatives inhibit bacteria by many different mechanisms (57–60). The PG, GO and GF nanomaterials of the electrospun nanocomposite fibers included in this study were mostly embedded in the fibers. Therefore, reductions in direct action mechanisms may occur with nanomaterials. While this result may increase the biocompatibility of the nanofibers used, it may cause a decrease in antimicrobial activity. The oxidative stress mechanism is one of the basic mechanisms of graphene-based nanomaterials (57). In addition, it is stated as another mechanism that the graphene nanomaterials suspended on the fiber surfaces cause the loss of substances inside the cell by direct contact and the effect of the microbial membrane of their sharp edges (35). Finally, another mechanism called wrapping is the model in which nanomaterials in the environment encapsulate and isolate microbes (58). In this study, PG and GF nanocomposite fibers might inhibit viruses with their nanosized pores, wrapping and 3D sharp edges, while GO might increase antiviral activity mostly with the oxidative stress effect. However, the viral mechanisms of graphene-based materials still are not clear enough and should be investigated in more detail.

In antibacterial nanofiber studies, GO was mostly preferred because of its oxidative stress physicochemical properties and high biocompatibility. However, in general, GO polymer nanocomposites, which exhibit dose-dependent and time-dependent antibacterial activities (20), are more efficient at higher concentrations. GO toxicity has been found to be safe within a certain range in the human body (61). In this study, graphene-based materials at selected concentrations are limited to a maximum concentration of 4 w/w% in biomedical research to prevent harm to humans. Compared with the antiviral activity of GO nanocomposite fibers in previous studies, 4 w/w%

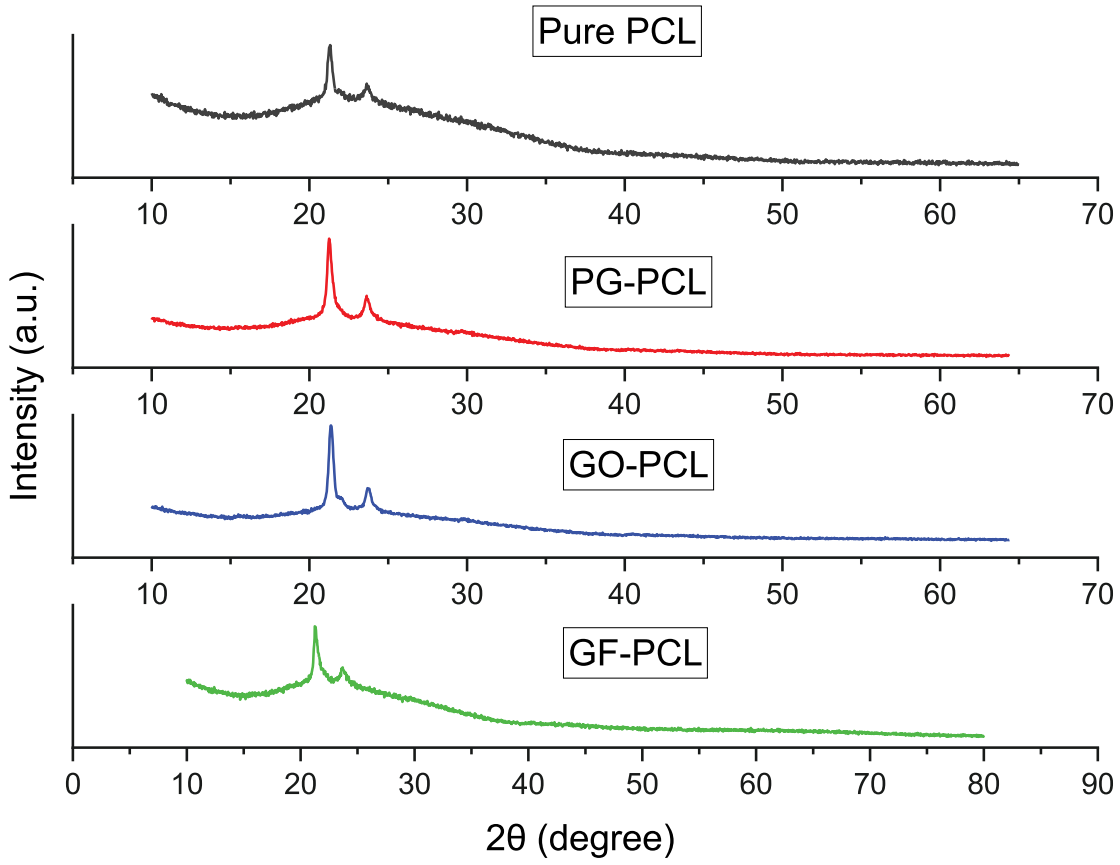

FIGURE 8
XRD analysis of pure PCL and 2w/w% PG-PCL, GO-PCL, GF-PCL nanofibers.

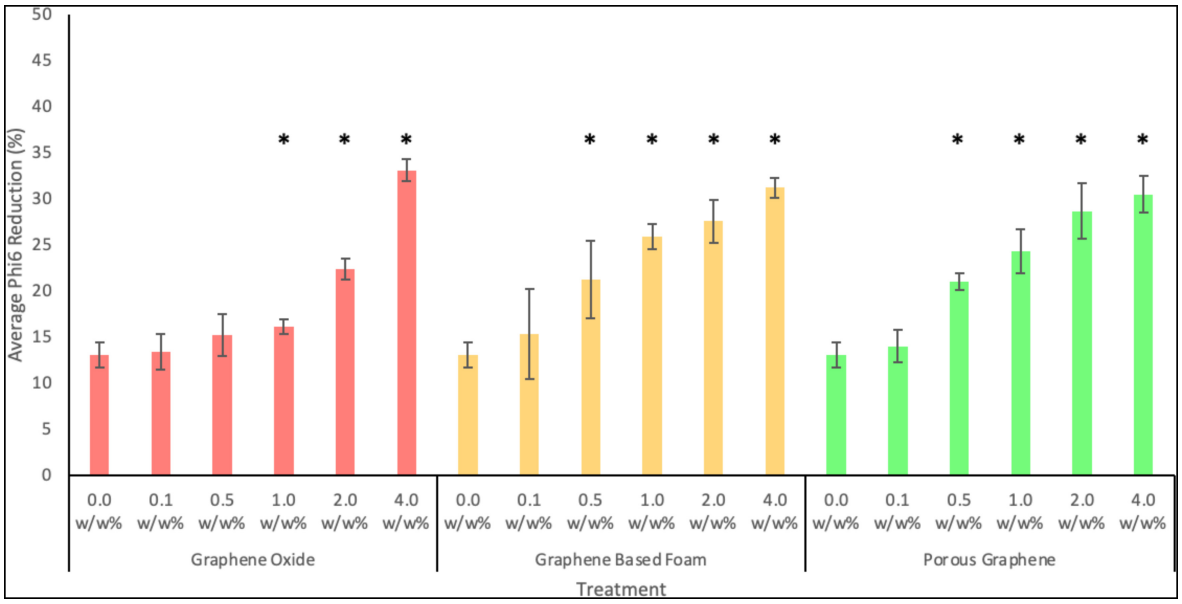

FIGURE 9
Antiviral activity of PCL fibers loaded with 0.1, 0.5, 1.0, 2.0 or 4.0 w/w% of GO, GF, or PG against Phi6 bacteriophage after 24 h. Error bars represent standard deviation (n = 3). P values of <0.05 compared to the control are indicated with an *.

GO was found to have a slightly higher antiviral effect in this study, 28.9 ± 1.2 and 33.08 ± 1.21%, respectively (18). The main reason for this can be explained as the fibers (0.53 ± 0.20 μm at 4 w/w% concentration) are thinner than in a previous study (1.55 ± 0.9 μm at 4 w/w% concentration) and therefore have a higher surface area to volume ratio (18). It is known that as graphene concentration of nanofibers increases, biocompatibility may decrease, and thus toxicity may increase.

The antiviral activity of GO has been investigated in previous studies (18), and during the COVID-19 pandemic graphene derivatives have been thought of as promising materials for the formation of antiviral fibrous mats. Even though this study was conducted against Phi6 bacteriophage, the data obtained indicate that graphene-based materials are potential antiviral candidates. Therefore, it has an important role for PPE used in preventing the spread of any viral infections.

Since PG, GO and GF nanomaterials are not completely soluble in acetone, they are dispersed and form a suspension. If the graphene derivatives in the formed polymer nanocomposite suspensions are not sufficiently dispersed, they may undergo agglomeration (39, 62). Agglomeration is seen in fibers with large bead formation. However, the reason for all of the beads formed may be not only due to the agglomeration of the particles but also to the electrospinning parameters. Another limitation is that nanomaterials are embedded in the fiber and not on the surface. Graphene-based material on the surface may show a higher antiviral effect, but for example, when used in face masks, it may also have a toxic effect as it can deposit in the lungs when inhaled directly. To prevent this, various surface modification methods such as electrospraying deposition can be tried.

Finally, electrospinning is a system that provides fiber formation from polymer solutions in the high electric field created by a high voltage power supply. At the same time, graphene and its derivatives have a high electroconductivity. Graphene-based materials in nanocomposite solution may be affected by the high voltage applied during nanofiber production and may not adhere to the fiber surface. In such cases, as the concentration may decrease, the antimicrobial activity will also decrease. At the same time, using the entire solution in the syringe in fiber formation is another important point. Since graphene is not completely dissolved in solution, it may collapse and remain in the syringe. This can likewise affect the amount of concentration. Therefore, it should be ensured that all the solution in the syringe and tube is used.

Conclusion

In this work, morphology, chemical analysis and the virus inhibitory properties of 0.1, 0.5,1, 2, 4 w/w% concentration PG, GO and GF-loaded fibers were compared. SEM, FTIR and XRD characterizations were applied to the nanofibers. According to the results, the ultrafine fibers obtained mostly have porous surface properties and the mean diameter of all fibers was measured at around 460 nm. It has been observed mostly that the general trend is an increase in nanofiber diameters as the nanomaterial concentration increases. PG, GO and GF nanofibers showed antiviral activity against the SARS-CoV-2 surrogate and the highest inhibition was recorded as 33.08 ± 1.21% after 24 h. In this present study, PG and GF antiviral properties were investigated for the first time. The data showed that overall graphene-based nanomaterials are promising for biomedical applications such as PPE against the ongoing COVID-19 pandemic.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

SGE performed conceptualization, resources, visualization, analysis, and wrote the original draft. TAT provided resources and characterization, also reviewed the original draft. ME was involved in the conceptualization and overall supervision, reviewed and edited the manuscripts. RKM wrote the original draft and was involved in the conceptualization and overall supervision, reviewed, and edited the manuscripts. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Intestinal colonization with ESBL-producing *Klebsiella pneumoniae* in healthy rural villager: A genomic surveillance study in China, 2015-2017

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Background: The worldwide emergence and diffusion of extended-spectrum β-lactamase-*K. pneumoniae* (ESBL-KP) is of particular concern. Although ESBL-KP can inhabit the human gut asymptomatically, colonization with ESBL-KP is associated with an increased risk of ESBL-KP infection and mortality. In this study, we investigated the prevalence and characteristics of ESBL-KP in fecal samples from healthy persons in 12 villages in Shandong Province, China.

Methods: Screening for ESBL-KP in fecal samples was performed by selective cultivation. The bacterial species were identified using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rDNA sequence analysis. Minimum inhibitory concentrations (MICs) of 16 antibiotics were determined by the agar dilution method. Plasmid replicons, antimicrobial resistance genes and Sequence types (STs) of the isolates were determined by whole-genome sequencing (WGS). Genetic relatedness of ESBL-KP isolates was determined by the single nucleotide polymorphisms (SNP). The S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) was used to characterize the plasmids carried by ESBL-KP isolates. Conjugation assays was used to verify the transferability of bla_{CTX-M}.

Results: ESBL-KP prevalence rates increased from 12.0% in 2015 to 27.5% in 2017. The experimental results showed that 97% of isolates had multi-drug resistance. Multiple ESBL resistance genotypes were commonly detected in the isolates. STs among the ESBL-KP isolates were diverse. All 69 $bla_{CTX-M-3}$-positive isolates were located on plasmids, and these genes could be transferred with plasmids between different strains. Phylogenetic analysis showed the possibility of transmission among some isolates.

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Conclusion: This study obtained the drug resistance patterns, the drug resistance phenotype and molecular characteristics of fecal-derived ESBL-KP in rural communities in Shandong Province, China. We report a rapid increase in occurrence of ESBL-KP among fecal samples collected from healthy rural residents of Shandong Province from 2015 to 2017. The carriage rate of multidrug-resistant bacteria in healthy residents is increasing. Thus, a need for further monitoring and possible interventions of ESBL-KP in this region is warranted.

KEYWORDS

intestinal colonization, ST101, CTX-M-3, core-genome single nucleotide polymorphisms, ESBL-producing *K. pneumoniae*

Introduction

ESBL-KP is one of the most common multi-drug resistant groups of Enterobacteriaceae worldwide (1). As an essential nosocomial pathogen, its invasive infection recently resulted in high mortality rates (2, 3). Acquired drug resistance in human commensal bacteria such as *Escherichia coli* and *K. pneumoniae* has become a widespread threat to public health (4). Of recent concern is the increasing prevalence and risk factors of ESBL-KP in community-acquired infections (2). ESBLs-producing is the main reason for *K. pneumoniae* developing drug resistance and spreading rapidly and widely. However, the exact situation of ESBL-KP in healthy residents living an ordinary life in the community is still unclear (5, 6).

The global epidemic of ESBLs mainly includes SHV, TEM, and CTX-M types, and in the last two decades, CTX-M has replaced SHV as the significant type of ESBLs disseminating worldwide (2, 7). More than 190 CTX-M variants have been reported worldwide to date (8). CTX-M-1 was the first reported CTX-M enzyme, and since then, many derivatives of CTX-M have been reported around the world. CTX-M-33, a point mutation derivative of CTX-M-15, was identified in *K. pneumoniae* from a patient hospitalized in Portugal. It is the first CTX-M enzyme possessing a weak carbapenemase activity (9). In Turkey, *E. coli* isolates from chicken meats were found to carry predominantly CTX-M-1 genes, followed by CTX-M-89 and CTX-M-2 (10). Recently, CTX-M-167 positive *K. pneumoniae* was identified for the first time in China (11). While $bla_{\mathrm{CTX-M-15}}$ and $bla_{\mathrm{CTX-M-14}}$ are identified as the most prevalent ESBL-encoding genes in the world, $bla_{\mathrm{CTX-M-14}}$ has been identified as the most common ESBL-encoding gene in China (12). The association of $bla_{\mathrm{CTX-M}}$ genes with mobile genetic elements, epidemic plasmids, and successful clones ensured rapid and wide dissemination that changed the epidemiology of antibiotic resistance worldwide (13).

Conjugative plasmids are one of the most important mechanisms for the appearance and spread of $bla_{\mathrm{CTX-M}}$. Additionally, these plasmids often encode multiple resistance determinants, including fluoroquinolones, quinolones, trimethoprim/sulfamethoxazole (SXT) and aminoglycosides (14). The study found three incompatibility (Inc) groups of plasmids, the narrow-host-range IncF and IncI1 plasmids and the broad-host-range IncC plasmid, formerly IncA/C2, are prevalent and associated with ESBL-producing Enterobacteriaceae strains (15). The plasmids carrying virulence genes and antimicrobial resistance may now pose a serious threat to public health because these mobile plasmids accelerate the horizontal transfer of these genes between strains of the same or different species (8, 16). Thus, the identification and characterization of these plasmids may facilitate a better understanding of ESBL-KP transmission mechanism and the development of new measures to control ESBL-KP producers in the community.

Our objectives in this study were to retrospectively investigate the drug resistance phenotype and molecular characteristics of human fecal-derived ESBL-KP in a rural community in Shandong Province, China, and to understand ESBL-KP in a Chinese rural community better. The prevalence of *Klebsiella* and its comparison with domestic and foreign genome-wide data were added to the current database. Our results will outline a larger picture of ESBL-KP within the Chinese community and will provide theoretical support for the government to establish a drug-resistant bacteria prevention and control system in the community.

Materials and methods

Bacterial isolation and identification

To better understand the prevalence of ESBL-KP, antimicrobial resistance of bacteria in feces of healthy adults was monitored in Shandong Province, China, in 2015 and 2017 (Figure 1). The selection of villages and participants has been described elsewhere (15). In 2015, 758 households in the study region were selected, and in 2017, 628 of these

households were sampled again. One individual from each household was sampled on each occasion. Fecal samples were collected and cultured overnight on chromogenic agar medium (ESBL-Bx, bioMérieux, Marcy l'Etoile, France) at 37°C to screen for ESBL-KP strains. The suspected *K. pneumoniae* colonies identified based on the manual's description were picked and subcultured on the corresponding basal agar at 37°C overnight. Identification of the bacterial species was identified based on 16S rDNA gene sequence analysis and MALDI-TOF MS, as described previously (12). SPSS 22.0 software was used for statistical analysis of the detection rate of each village, and CohranMantel Haenszel was used for stratification Chi-squared test for comparison between groups, $P < 0.05$ indicated that the difference was statistically significant.

Antimicrobial susceptibility testing

A total of 16 antibiotics were tested. MICs of 14 antimicrobial agents (cefotaxime, ceftazidime, cefoxitin, piperacillin-tazobactam, gentamicin, amikacin, amoxicillin-clavulanate, trimethoprim-sulfamethoxazole, imipenem, meropenem, tetracycline, florfenicol, and furantoin) were determined by agar dilution method, and MICs of two other antimicrobial agents (tigecycline and colistin) were determined by the broth microdilution method. The breakpoints for susceptibility were determined according to Clinical & Laboratory Standards Institute (CLSI, USA) and European Committee on Antimicrobial Susceptibility Testing (EUCAST, Europe). ESBL phenotype was confirmed by the double-disk diffusion method with incorporate cefotaxime and ceftazidime tested alone and combined with clavulanate, according to CLSI. The above experiments used *E. coli* ATCC25922 as quality control (12). Chi-squared test was used for statistical analysis to compare the difference of drug resistance between the 2 years.

Whole-genome sequencing

All ESBL-KP strains were subjected to WGS. Genomic DNA was extracted using a commercial kit. Libraries were prepared from genomic DNA according to the manufacturer's protocol and sequenced on an Illumina HiSeq (paired end, 2 × 150 bp). *De novo* assembly was generated by using SPAdes 3.11.0 (17). Plasmid replicons, antimicrobial resistance genes and STs were predicted from the genomes using PlasmidFinder 1.3, ResFinder 2.1, and MLST 2.0, respectively (18). The genetic environment surrounding $bla_{\mathrm{CTX-M}}$ was annotated using RAST3 and Easyfig 2.2.3 (19). In order to determine the phylogenetic relationships among study isolates, and compared them with isolates from diverse host sources from other regions to place them in the international context. For this purpose, we downloaded genome sequences of 99 ESBL-KP isolates from various regions of the world. Using the kSNP3 analysis software, the kmer_length parameter was set to 13 for SNP analysis and phylogenetic tree construction, and evolview beautified and modified the phylogenetic tree (20).

S1 nuclease-pulsed-field gel electrophoresis (S1-PFGE) and Southern blotting

The CTX-M-3 enzyme has been widely detected; however, its genetic environment has rarely been well investigated as other prevalent ESBLs. S1-PFGE and Southern blotting were performed to locate $bla_{\mathrm{CTX-M-3}}$ and determine plasmid size in all $bla_{\mathrm{CTX-M-3}}$-positive *K. pneumoniae*. Whole-cell DNA of all $bla_{\mathrm{CTX-M-3}}$-positive isolates was extracted and embedded in agarose gel plugs. The DNA in agarose plugs was treated with S1 nuclease (TaKaRa, Beijing, China), and separated by PFGE. Plasmids obtained by PFGE were transferred horizontally to a nylon membrane and hybridization with labeled probes in an HL-2000 HybriLinker Hybridization Oven (UPV, Germany) (12). Molecular mass standard reference strain was *salmonella enterica* serotype Braenderup strain H9812.

Conjugation assay

Transfer of antibiotic resistance was studied using conjugation for all ESBL-KP isolates. *E. coli* EC600 (rifampicin-resistant) or *E. coli* J53 (azide-resistant) was used as a recipient strain, whereas all ESBL-KP resistant to cefotaxime served as donors. The experimental details were described in our previous study (12).

Nucleotide sequence accession number

This whole genome and annotation has been deposited in NCBI GenBank under the accession number of PRJNA833746.

Results

ESBL-KP isolations and isolation sites

ESBL-KP was isolated from 91 and 173 individuals in 2015 and 2017, respectively. This corresponds to an increase in ESBL-KP occurrence from 12.0% in 2015 to 27.5% in 2017. The mean age of the study participants was 66 with a range between 20–85 years old, and 53% ($n = 22$) were women. The detection rate of ESBL-KP increased in all but three villages. There were statistical differences in the trend of the detection rate among different villages with the year ($\chi^2 = 24.682$, $P = 0.010$) (Figure 1).

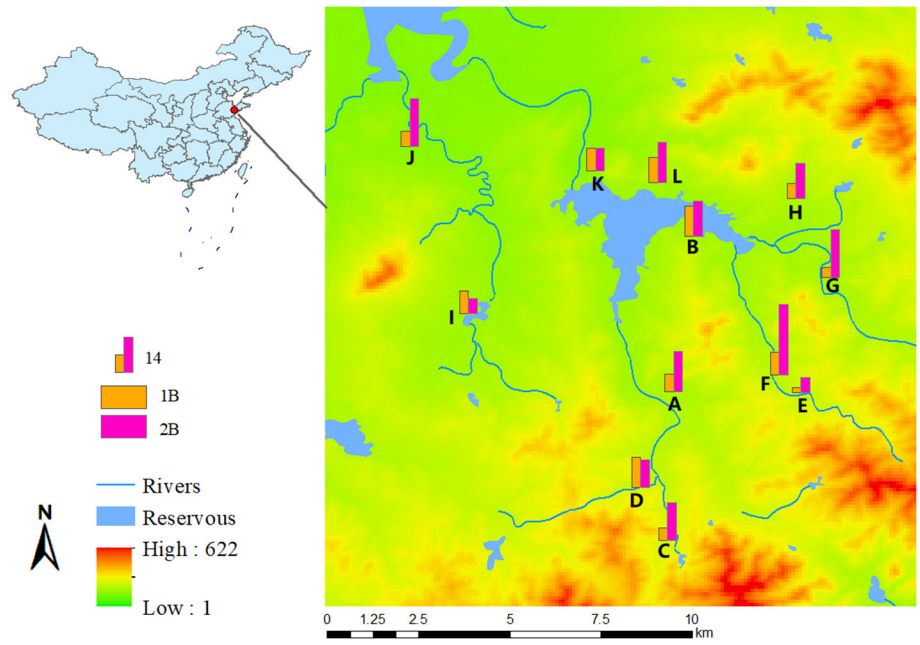

FIGURE 1
Geographic distribution map of ESBLs-producing *K. pneumoniae* from village A to L, in 2015 and 2017, with digital elevation model.

Antimicrobial resistance profiles of ESBL-KP isolates

All isolates were insensitive to cefotaxime, tetracycline and florfenicol. In addition, susceptibility to ciprofloxacin and trimethoprim-sulfamethoxazole was also lower (< 10%), while susceptibility to meropenem, amikacin, and tigecycline was higher (≥ 90%). Statistically significant differences were observed in susceptibility rates for ciprofloxacin and tetracycline ($p < 0.05$) in 2015 and 2017. The isolates were also generally sensitive to amikacin (95.6%, 98.3%), piperacillin-tazobactam (98.9%, 98.3%), ceftazidime (93.4%, 90.8%), cefoxitin (98.9%, 95.4%) and Colistin (91.2%, 94.02%), however showed lower sensitivity to trimethoprim-sulfamethoxazole (7.7%, 3.5%) (Figure 2). Moreover, the experimental results showed that 97% of isolates have multi-drug resistance (i.e., resistant to antibiotics of more than two classes of antibiotics).

ESBL-encoding genes

Whole-genome sequencing showed that all ESBL-KP isolates carry $ESBL_S$ resistance genes. Various TEM, SHV, and CTX-M types were commonly detected in isolates. The most common ESBL genes were bla_{TEM-1B} (124, 47.0%), $bla_{CTX-M-14}$ (112, 42.4%), and bla_{SHV-11} (86, 32.6%) in all isolates in 2015 and 2017. The coexistence of CTX-M, SHV, and TEM genes was identified in 115 isolates (43.6%). In addition, both CTX-M and SHV co-existed in 103 isolates (39.0%), and both CTX-M and TEM co-existed in 14 isolates (5.3%).

The bla_{CTX-M} genes were detected in 230 (87.1%) of 264 ESBL-KP isolates. The $bla_{CTX-M-14}$ gene was the most prevalent one ($n = 114$), followed by $bla_{CTX-M-3}$ ($n = 70$), $bla_{CTX-M-15}$ ($n = 15$), $bla_{CTX-M-27}$ ($n = 14$), $bla_{CTX-M-24}$ ($n = 7$), and $bla_{CTX-M-65}$ ($n = 5$), $bla_{CTX-M-55}$, and $bla_{CTX-M-5}$ ($n = 2$ for each), and $bla_{CTX-M-105}$ ($n = 1$). Additionally, both bla_{CTX-M} genes co-existed in 5 isolates. The detection of ESBL genes in ESBL-KP in 2015 and 2017 is shown in Figure 3.

Non-ESBL antimicrobial resistance genes

Plasmid-mediated quinolone resistance (PMQR) genes were identified in all isolates. Apart from *oqxA* and *oqxB*, which were detected in all except four isolates (98.5%), and-type genes (including *qnrB1, qnrB2, qnrB4, qnrB6, qnrB49, qnrB52, qnrB66, qnrS1* and *qnrS2*) were detected in 251 isolates (95.1%), with *qnrS1* being the most abundant and found in 212 isolates (80.3%). Sulphonamide resistance genes were present in 97.7% ($n = 258$) of isolates, with the *sul1* gene as the most prevalent (259, 98.1%). The *sul1* and *sul2* genes often co-occur in the same strain in this study, and the *sul3* gene was detected in three isolates in 2015. Aminoglycoside resistance genes were commonly detected, and 252 isolates (95.5%) carried at least one aminoglycoside resistance gene.

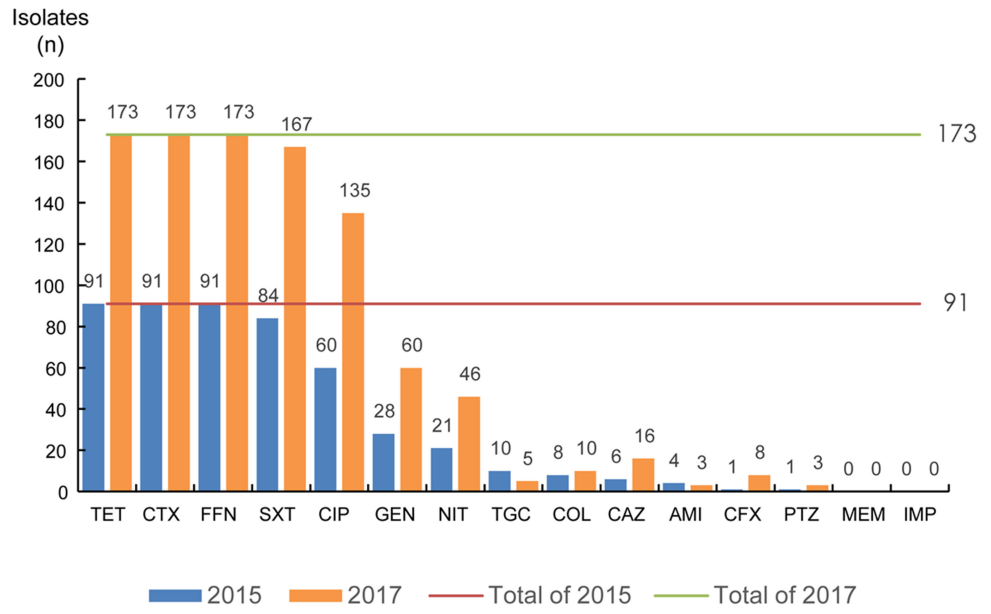

FIGURE 2
The antimicrobial susceptibility of ESBLs-producing *K. pneumoniae*. TET, tetracycline; CTX, cefotaxime; FFN, florfenicol; SXT, trimethoprim-sulfamethoxazole; CIP, ciprofloxacin; GEN, gentamicin; NIT, furantoin; TGC, tigecycline; COL, colistin; CAZ, ceftazidime; AMI, amikacin; CFX, cefoxitin; PTZ, piperacillin-tazobactam; MEM, meropenem; IMP, imipenem.

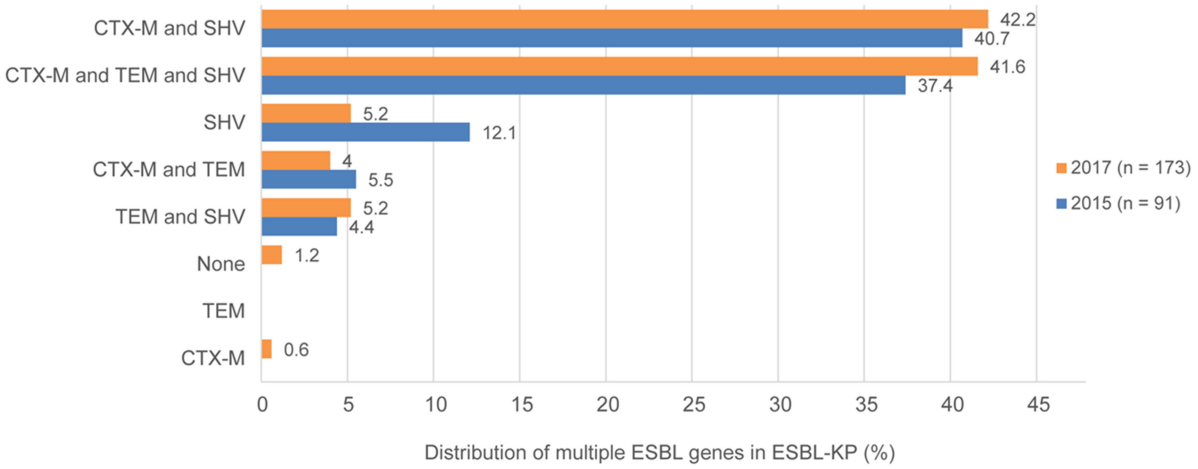

FIGURE 3
Existence and proportions of multiple ESBL genes for ESBLs-producing *K. pneumoniae* in 2015 and 2017.

The most common were *aac(3)-IId* (150, 56.8%), followed by *aac(6')-Ib-cr* (113, 42.8%), *aph(3')-Ib* (68, 25.8%), *aadA16* (107, 40.5%), and *aph(3')-Ia* (69, 26.1%). Notably, the linked strA-strB genes, which confer streptomycin resistance in at least 17 genera of gram-negative bacteria, were detected in 51 isolates (19.3%). A total of 217 isolates were found to carry *tet(A)* (82.2%), followed by *tet(D)* (74, 28.0%) and *tet(G)* (1, 0.38%). All of the isolates carrying *tet*-type genes were resistant to tetracycline. Furthermore, the colistin resistance gene *mcr-1* gene was detected in one isolate recovered in 2015.

Multilocus sequence determination of the ESBL-KP isolates

The ESBL-KP collected in the study showed high diversity in terms of STs. The 264 isolates displayed 105 different sequence

TABLE 1 Analyses of the plasmids for *bla*$_{CTX-M-3}$-positive *K. pneumoniae*.

Plasmid replicon type[a]	Number of isolates (*n*)	size approximation (kb) (*n*)
IncFII(K)	41	216.9 (2), 138.9 (11), 104.5 (21), 78.2 (7)
Col4401	10	138.9 (7), 104.5 (3)
IncQ1	11	104.5 (8), 78.2 (3)
IncFIB(K)	3	173.4 (1), 104.5 (2)
IncFII(pKP91)	1	173.4
IncFII(pKPHS1)	1	138.9
IncR	1	138.9
IncFIIB	1	104.5

[a]Types of plasmid origins based on the PlasmidFinder analysis of the assembled genome sequences.

types (ST), and only 9 STs were detected in isolates from both years. ST101 is the most common STs (21, 8.0%), ST37 (16, 6.0%), and ST661 (8, 3.0%) and the three ST isolates together constituted 17.13% of all isolates. These STs were followed in prevalence by ST17, of which seven isolates belonged (2.7%), and ST323, ST896, ST34 and ST1263, for which six isolates of each ST were found (2.2%). Notably, most of the detected STs corresponded to only one isolate representative ($n = 61$).

Determination of the *bla*$_{CTX-M-3}$ gene location and transferability

In this study, S1-PFGE has confirmed this multi-plasmid nature in at least 35 strains (Figure 5). The Southern blot hybridization demonstrated that the *bla*$_{CTX-M-3}$ genes among all 69 *bla*$_{CTX-M-3}$-positive isolates were located on plasmids, the type and size of the plasmid where A is located are shown in Table 1. Interestingly, the *bla*$_{CTX-M-3}$ gene of two isolates was located on two plasmids of different sizes, respectively (Table 1, Figure 5). Furthermore, all *bla*$_{CTX-M-3}$ genes could be transferred from the isolate to the recipient by conjugation, and this gene in the recipient was detected by PCR (data not shown).

Genetic context of ESBL genes

The *bla*$_{CTX-M-3}$ gene and *bla*$_{CTX-M-14}$ gene surrounding the different isolates showed exactly the same genetic background. The *bla*$_{CTX-M-14}$ gene was flanked by an IS*E062*, IS*1400* and IS*Ecp1* elements upstream and a partial TnAs1 downstream. The presence of the beta-lactam gene (*bla*$_{TEM}$) and tetracycline resistance gene (*tet*) downstream of *bla*$_{CTX-M-3}$. However, genetic variations between *bla*$_{CTX-M-15}$ encoding elements were frequent due to different lengths of the IS*Ecp1* elements, different sizes or locations of the region between IS*Ecp1* and *bla*$_{CTX-M-15}$, and/or a different length of the partial elements (IS*150IV* and Tn*2*). Three distinct gene environments were displayed around the *bla*$_{CTX-M-55}$ gene, whereas ISEcp1 was all located downstream of the gene (Figure 4). Overall, isolates with the same genetic environment appeared in different villages at different times, suggesting the possibility of human-to-human transmission of plasmids carrying this gene.

Characterizing ESBL-KP SNPs and phylogeny

The results of the SNP analysis showed that there is a possibility of ESBL-KP transmission in the community. For example, ST659 ESBL-KP isolate 2BC013 recovered in Village C was closely related to 2BC023 retrieved from the same village in 2017 (15 SNPs difference), ST323 ESBL-KP 2BB035 recovered from Village B was closely related to 2BL048 from Village L in 2017 (one SNPs difference), ST776 ESBL-KP 2BF007 and 2BF015 separated in different households from village F in 2017 were closely related (78 SNPs difference). Further, potential ESBL-KP close associations were also detected between individuals in various villages at different times, such as isolates 1BL052 (ST869) from L village and 2BH029 (ST869) from H village (SNPs difference of 5), 1BK025 (ST101) from village K and 2BH055 (ST101) from village H (5 SNPs difference), 1BK025 (ST101) from village K and 2BH055 (ST101) from village H (5 SNPs difference). Surprisingly, no close correlation was found for ESBL-KP from the same villagers. In addition, we compared genome sequences of these isolates to selected ESBL-KP isolated from food, animal, environment and human sources available on the public data base. ST442 ESBL-KP isolates 2BA003 recovered from village A were closely related to the United Kingdom human-derived sample isolate VRC00451 (99 SNPs difference), the remaining 98 isolates were not closely genetically related to the isolates in the study (between 176 and 2098 SNPs) (Figure 6).

Discussion

Worldwide, the resistance of ESBL-KP to many antibiotics has become a major global problem, which has seriously affected the treatment of infectious diseases (2, 21). In Latin America, ESBLs are produced in approximately 25% of *E. coli* and 53% in *K. pneumoniae*. In Chile, however, the incidence of ESBLs produced by *K. pneumoniae* strains resistant to third-generation cephalosporins is approximately 60% (22, 23). The carrier rate of

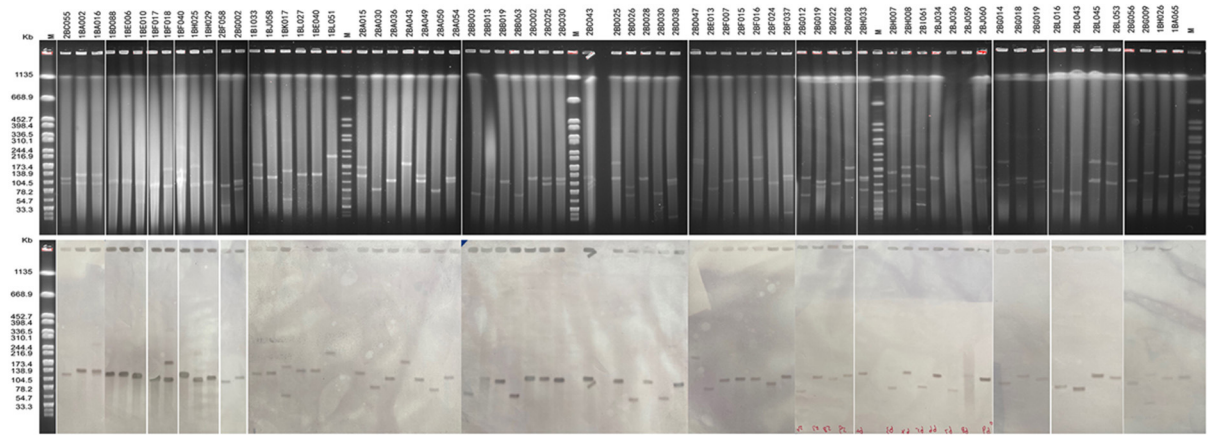

FIGURE 4
S1-PFGE analysis of $bla_{CTX-M-3}$-positive *K. pneumoniae* isolates and southern hybridization. Lane M, *Salmonella* Braenderup H9812 as molecular weight marker.

ESBL-producing *E. coil*/*K. pneumoniae* among veterinarians in the Netherlands is 9.8% (24). In China, the overall positive rate of ESBLs in Enterobacteriaceae from patients with nosocomial urinary tract infection was 37.2% (25). Studies have reported that colonization rates of ESBL-producers are rising in patients and health care worker populations, thereby increasing the size of the repository and increasing the chances of transmission (26, 27). In this study, we used a WGS approach to analyze 264 ESBL-KP isolates collected in two years (2015 and 2017) from the same rural community in China, an Asian country from which data such as these are acutely lacking (19).

In general, there was a correlation between phenotypic resistance and related genes of ESBL-KP strains isolated in this study. These strains isolated carried a large number of antibiotic resistance genes (Figure 7), including quinolones, aminoglycosides, sulphonamides, trimethoprims, tetracyclines, beta-lactams, phenicols and fluoroquinolones, and it was found that the isolates carrying these resistance genes produced different degrees of resistance to related drugs. All isolates carried quinolone resistance genes, but 26.5% of isolates were not resistant to such drugs, which may be related to the low level of gene expression. Comparing 2015 and 2017, susceptible rates of gentamicin, cefotaxime, ciprofloxacin, ceftazidime, furantoin and trimethoprim-sulfamethoxazole decreased slightly, while susceptible rates of amikacin and tigecycline increased, with the tigecycline increasing rate from 10.90 to 3.80% (Figure 1). Overall, ESBL-KP isolates showed a downward trend in antimicrobial susceptibility. In addition to cefotaxime, susceptible rates were also low (<10%) to trimethoprim-sulfamethoxazole, florfenicol and tetracyclines. Non-susceptibility to these antibiotics corresponded well to carriage of trimethoprim (*dfr*-type), sulphonamide (*sul*-type), phenicol (*floR*-type) and tetracycline resistance genes (tet-type).

All ESBL-KP isolates in this study were not susceptible to florfenicol. Florfenicol is a broad-spectrum antimicrobial exclusively used in veterinary medicine (28). It is still banned for use in humans. The widespread use of florfenicol has led to the emergence and development of cross-resistant pathogens that may enter the human body through the food chain (29). Recently, many florfenicol-resistant bacteria and florfenicol-related resistance genes (*floR*) have been isolated from various animal, human and environmental samples. It is, therefore, speculated that the carriage of this gene, and the lower susceptibility rates, may also be sustained by a selection pressure induced on animals and subsequent run-off into the environment (30, 31). MCR-1 was identified in an ESBL-KP isolate from 2015. The association of *mcr-1* with other resistance mechanisms, such as the production of ESBLs and carbapenemases, which has lead to the development of multidrug-resistant bacteria, could represent a serious clinical problem today (32, 33). The Chinese government has banned the use of polymyxins in animal feed since late 2016 (34). This may be one of the reasons why *mcr-1* was not found in ESBL-KP isolates in 2017 (33).

ST101 has the potential to pose a persistent threat to global public health as an emerging clone that has been identified in many countries and regions. Recent studies have shown that ST101 lineage infection causes an 11% increase in mortality compared with non-ST101 lineage (35). Studies have shown that ST101, a new emerging and extensively resistant sequence type, can further enhance the high risk spreading of carbapenem resistance spreading and polymyxin resistance (36–38). *K. pneumoniae* isolates of type ST101

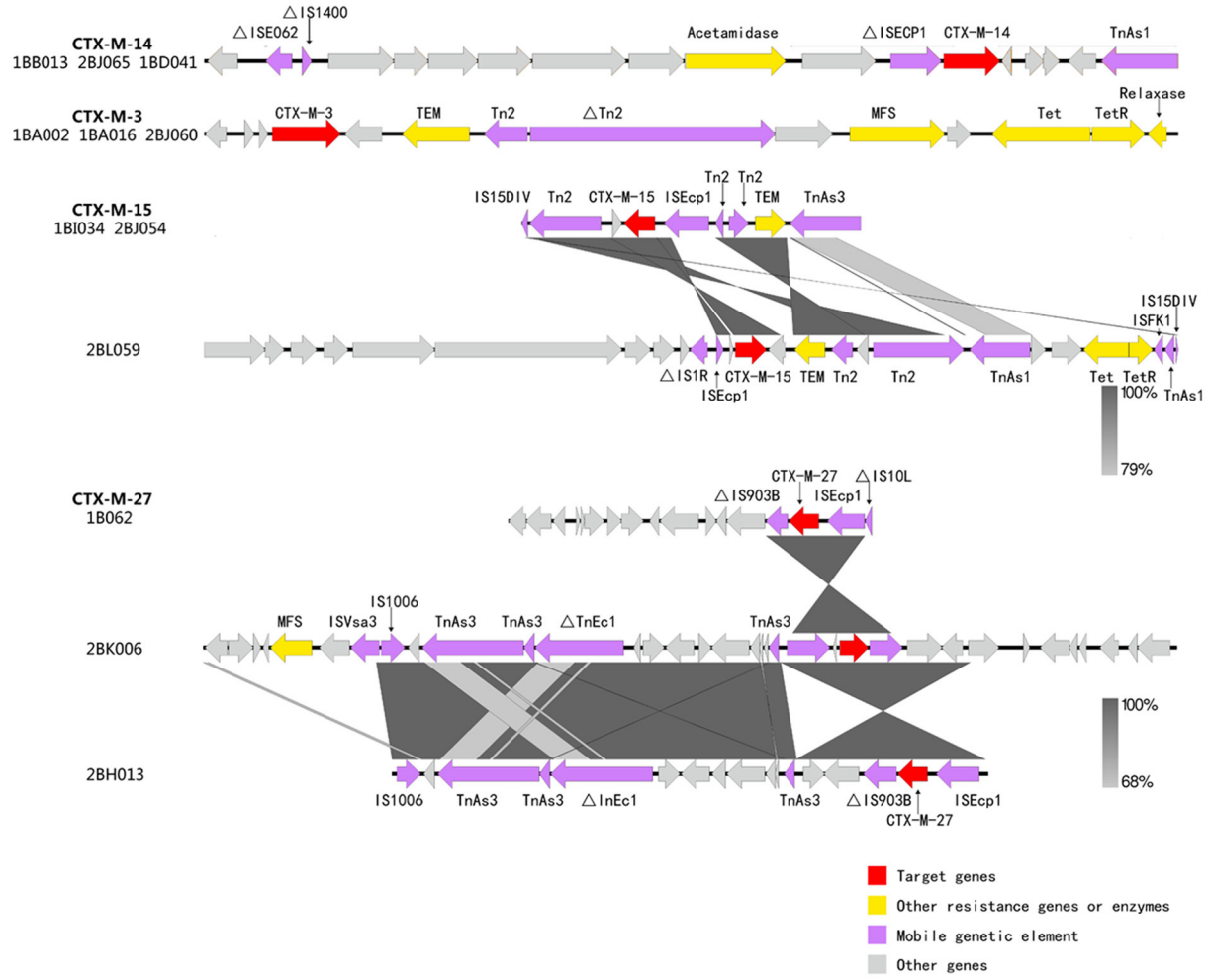

FIGURE 5
Analysis of the genetic environment surrounding the *bla*$_{CTX-M}$ gene. Arrows represent the direction of transcription. Red filled blocks represent CTX-M, yellow filled blocks represent resistance-related proteins, purple filled blocks represent mobile genetic elements, and gray filled blocks represent other proteins.

are closely associated with hospital-acquired infections and epidemics worldwide (39). ST101 and ST274 clones were predominant in ESBL-KP from domestic pets in Italy and France, but ST15 was the most common among ESBL-KP clinical strains from domestic pets in Japan (40). ST3330 clones were dominant in ESBL-KP from an outbreak of sepsis among neonates in an intensive care unit of a China hospital (41). Compared with ESBL-KP obtained in Chinese hospitals and communities, ESBL-KP of healthy people in rural communities showed a different clone distribution. A study from a Chinese hospital showed that ST11 was the most detected ST type in 158 ESBL-KP (42). Another study in a Chinese hospital showed that ST17 is the most popular ST among community-acquired ESBL-KP (2). However, ST101 was the most prevalent one in our study (8.2%). Therefore, the predominant ST in ESBL-KP varies by source sample type and country.

CTX-M-14 and CTX-M-3 were the EABLs with the highest detection rates in our study. This is similar to CTX-M types of ESBL-producing *Raoultella ornithinolytica* and ESBL-KP isolated in environmental samples from the same region (12, 19). This may indicate that bacterial resistance is not only transmitted between the environment and human hosts but also between different bacterial species. The *bla*$_{CTX-M}$ gene has been found on some plasmid types such as InCHI2, IncK, IncF, ZSH6, IncP, IncM2, IncN, IncI and IncL/M (2, 43–45). Our annotated ESBL-KP genome identified incompatibility (Inc) groups of plasmids for IncFIB(pKPHS1), IncFII(K), IncFIA(HI1), IncHI1B, IncHI2A, IncQ1 and IncR. Conjugative plasmids are considered to be one of the

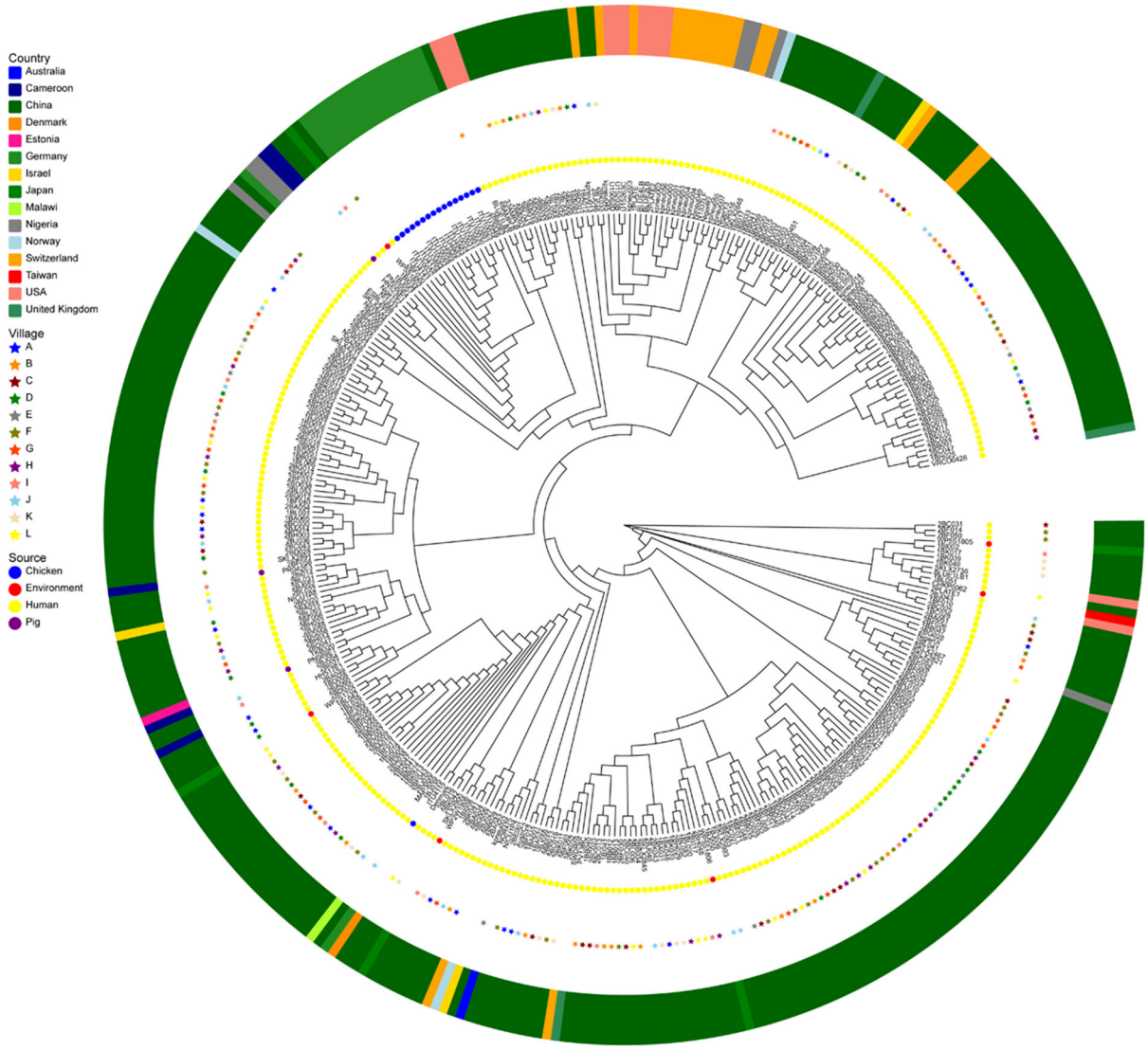

FIGURE 6
Genomic analysis of ESBLs-producing *K. pneumoniae* ($n = 264$) of this study and *K. pneumoniae* (n = 91) from various regions of the world ($n = 91$). A maximum-likelihood phylogenetic tree was constructed using genome-wide single-nucleotide polymorphism (SNP) analysis. Sources of the isolates are indicated by different colors for location (asterisks or squares) and samples' origin (circles). This core-genome-based maximum-likelihood tree was visualized ignoring information in gene-tree branch lengths for the convenience of discrimination.

important factors for the successful spread of CTX-M ESBLs among various bacteria such as *K. pneumoniae* (46). The hybridization results showed that 93.4% ($n = 54$) of the $bla_{CTX-M-3}$ gene was carried by the 78.2–138.9 kb IncF plasmid (Figure 5). The transferability of the plasmids in this study hints at their potential transmission among different host bacteria, and also, due to their wide resistome, this high-risk plasmid may lead to limited treatment options for infected individuals. BLAST analysis showed three isolates (1BB013, 2BJ065 and 1BD041) from different villages at different times showed a similar sequence around $bla_{CTX-M-14}$, i.e., ΔIS*E062*-ΔIS*Ecp1*-$bla_{CTX-M-14}$-Tn*As1*, which could be related to the lateral transfer of the genetic elements or the clonal expansion of the original host bacteria by some way during this time period. Upstream or downstream of $bla_{CTX-M-15}$ and $bla_{CTX-M-27}$, insertion of several mobile genetic elements (ISEcp1, IS15DIV and/or IS1006) takes place within Tn2/TnAn3, resulting in truncation of a portion of Tn2/TnAn3 with the blaTEM genes remaining in the loci. The IS insertion may introduce foreign resistance genes into the gene structure, such as tetracycline resistance genes (*tet*) and phenicol resistance genes (*flor*), which we speculate is the

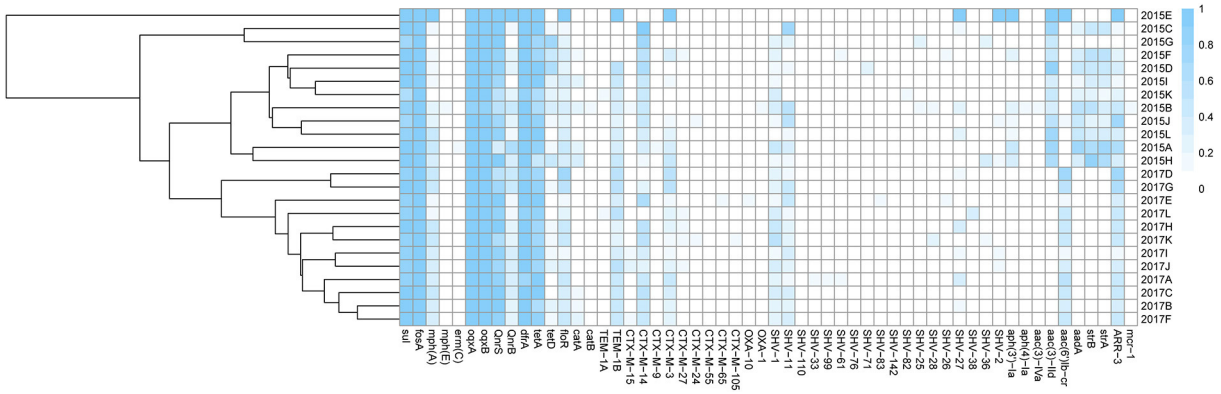

FIGURE 7
Distribution of phylogenetic group, AMR among ESBL-KP isolates from 12 villages in Shandong Province, China. The color of each box represents the percentage of the resistant gene among sequenced isolates in the corresponding village of the 2 years.

result of some antibiotic selection pressure (e.g., florfenicol) (Figure 4).

Our study has several limitations. First, the lack of timely analysis of the isolates in this study led to a long research period. Second, the number of ESBL-KP genomes downloaded from the database was small, so no strains closely related to the genetic relationship of the studied isolates were found.

Altogether, we characterized ESBL-KP strains isolated from the gut of healthy humans in terms of their resistance, resistance genes, and plasmid-mediated transmission mechanisms. Almost all ESBL-KP are multidrug-resistant. All $bla_{\mathrm{CTX-M-3}}$ were located on the plasmid of ESBL-KP isolates, and the plasmid could transmit between strains from different sources, which presents a new challenge for interrupting the transmission of ESBL-KP in the future. Therefore, it is necessary to further clarify the transmission route of ESBL-KP in this region, strengthen community dialogue, and study the potential health risks associated with it.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA833746.

Ethics statement

The studies involving human participants were reviewed and approved by Shandong Provincial Center for Disease Control and Prevention Medical Ethics Committee Shandong Provincial Center for Disease Control and Prevention. The patients/participants provided their written informed consent to participate in this study.

Author contributions

SW: field investigation, data curation, formal analysis, and writing–original draft. ZK: supervision and writing–reviewing and editing. HZ: supervision and funding acquisition. HX: field investigation, validation, and methodology. YC: field investigation, methodology, and software. MF: software and formal analysis. LL: methodology and software. DS: resources and formal analysis. LX, GS, and YL: field investigation. ZB: conceptualization. XY: methodology. BZ: conceptualization, supervision, funding acquisition, and writing-reviewing and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The resistance mechanisms of bacteria against ciprofloxacin and new approaches for enhancing the efficacy of this antibiotic

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For around three decades, the fluoroquinolone (FQ) antibiotic ciprofloxacin has been used to treat a range of diseases, including chronic otorrhea, endocarditis, lower respiratory tract, gastrointestinal, skin and soft tissue, and urinary tract infections. Ciprofloxacin's main mode of action is to stop DNA replication by blocking the A subunit of DNA gyrase and having an extra impact on the substances in cell walls. Available in intravenous and oral formulations, ciprofloxacin reaches therapeutic concentrations in the majority of tissues and bodily fluids with a low possibility for side effects. Despite the outstanding qualities of this antibiotic, *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli,* and *Pseudomonas aeruginosa* have all shown an increase in ciprofloxacin resistance over time. The rise of infections that are resistant to ciprofloxacin shows that new pharmacological synergisms and derivatives are required. To this end, ciprofloxacin may be more effective against the biofilm community of microorganisms and multi-drug resistant isolates when combined with a variety of antibacterial agents, such as antibiotics from various classes, nanoparticles, natural products, bacteriophages, and photodynamic therapy. This review focuses on the resistance mechanisms of bacteria against ciprofloxacin and new approaches for enhancing its efficacy.

KEYWORDS

review, ciprofloxacin, resistance, new approach, antibacterial agents

Introduction

A member of the fluoroquinolone (FQ) family of antibiotics, ciprofloxacin can be used to treat a variety of Gram-positive and Gram-negative bacteria. FQs regulate bacterial DNA supercoiling, a procedure necessary for DNA replication, recombination, and repair, by binding to and inhibiting DNA gyrase enzymes. The United States Food and Drug Administration (FDA) has given the drug approval for the treatment of gastrointestinal and lower respiratory tract infections, anthrax, plague, salmonellosis, skin, bone, and joint infections, prostatitis, typhoid fever, and sexually transmitted infections like gonorrhea and chancroid. It has also been recommended by World Health Organization (WHO) for treating tuberculosis (TB) as the second-line treatment for multidrug-resistant (MDR) TB (1, 2).

Nonetheless, there are increasing reports of ciprofloxacin resistance in *Bacillus anthracis*, *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Enterococci*, *Escherichia coli*, and *Klebsiella pneumoniae* (3, 4). The resistance could develop by efflux pumps or mutations in DNA gyrase genes (*gyrA*) (3, 5). Ciprofloxacin can also be used in the treatment of malaria (6). In this regard, the review mainly concentrated on the various properties of ciprofloxacin, its clinical applications for the treatment of different microbial infections, and bacterial resistance mechanisms to this antibiotic, as well as new strategies for enhancing ciprofloxacin efficacy against MDR bacteria.

Ciprofloxacin characteristics

Structure of drug

One-cyclopropyl-6-fluoro-4-oxo-7-(piperazine-1-yl)-1, 4-dihydroquinoline-3-carboxylic acid is the molecular name for the antibiotic (6). Its molecular weight is 331.34 g/mol and its chemical formula is $C_{17}H_{18}FN_3O_3$ (7). A quinolone, quinolin-4(1H)-one is the name of the antibiotic, and it has the functional groups cyclopropyl, carboxylic acid, fluoro, and piperazin-1-yl at positions 1, 3, 6, and 7, respectively (Figure 1) (7). The fluorine group at position C-6 and the piperazine group cause the expansion of the antimicrobial spectrum of ciprofloxacin. The piperazine group, also found in cefoperazone and piperacillin, increases ciprofloxacin activity against *Pseudomonas*. The cyclopropyl group is related to the high antibacterial activity of ciprofloxacin (8).

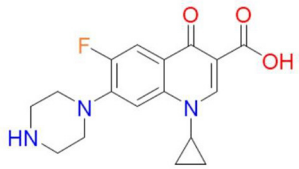


FIGURE 1
Chemical structure of ciprofloxacin (9).

Pharmacokinetics and pharmacodynamics

The pharmacokinetic profile of ciprofloxacin has been investigated for absorption, distribution, metabolism, and clearance. Studies have been performed by testing healthy and patient volunteers. Ciprofloxacin is absorbed fast and well and penetrates the tissues very well after oral administration. It shows gastrointestinal absorption and bioavailability range between 60 and 85% (8). Time to the maximum concentration of drug in serum (T_{max}) was approximately between 40 to 80 min, and the maximum concentration of drug in serum (C_{max}) was around 1 mg/L for a dosage of 200 mg. In a comparison between fasting and non-fasting volunteers, it was found that fasting volunteers showed higher C_{max} and shorter T_{max} than non-fasting volunteers, which means the presence of food interferes with the absorption of ciprofloxacin *.

Ciprofloxacin has low serum binding protein; it shows a mean protein binding of 39% in 0.5, 1, 2, and 5 mg of ciprofloxacin per liter. Ciprofloxacin has great distribution and tissue penetration; accordingly, drug concentration in most tissues and body fluids is higher than in serum (8). Ciprofloxacin can be metabolized in four ways: the primary ways are oxo-ciprofloxacin and sulfo-ciprofloxacin, and two minor ways are ethylene ciprofloxacin and formyl-ciprofloxacin; they are excreted by urine and feces. Unchanged ciprofloxacin was the major molecule appearing in the urine and feces (5).

Mechanism of action

Ciprofloxacin is a broad-spectrum antibiotic that affects its target by inhibiting the DNA gyrase, which is known as topoisomerase II and topoisomerase IV (10). DNA gyrase contains subunits A and B. Quinolones such as ciprofloxacin are believed to prevent subunit A from resealing the DNA

Abbreviations: FQs, fluoroquinolones; FDA, Food and Drug Administration; UTI, urinary tract infection; WHO, World Health Organization; TB, tuberculosis; MDR, multidrug-resistant; *gyrA*, DNA gyrase genes; QRDR, quinolone resistance determining region; PMQR, plasmid-mediated quinolone resistance gene; MIC, minimal inhibitory concentrations; SNPs, single nucleotide polymorphisms; MRSA, methicillin-resistant *Staphylococcus aureus*; MBC, minimum bactericidal concentration; PDT, photodynamic therapy; ROS, reactive oxygen species; MB, methylene blue; LPS, lipopolysaccharide structure.

double-strand; therefore, single-stranded DNA may result in exonucleolytic degradation (5). In most studies, the effect of ciprofloxacin on DNA gyrase has been emphasized; however, a previous investigation has suggested that ciprofloxacin could affect *Mycobacterium smegmatis* cell wall compounds. It has also been demonstrated that ciprofloxacin, in addition to its effect on DNA gyrase, can cause reduction in the amount of DNA, RNA, and protein, as well as phospholipids, galactose, arabinose, glucosamine, and the mycolic acid of the *M. smegmatis* cell wall. However, these findings should be confirmed in the further studies (Figure 2) (11). Ciprofloxacin affects several Gram-positive bacteria such as *Staphylococcus*, *Streptococcus*, *Enterococcus*, *Bacillus* spp., and *Mycobacterium*. Furthermore, ciprofloxacin shows an acceptable *in vitro* activity against most Gram-negative bacteria strains such as most species of *Enterobacteriaceae*, *N. gonorrhoeae, Neisseria meningitides, Haemophilus influenza, Moraxella catarrhalis*, *P. aeruginosa*, and *Legionella* species (5, 12). According to a study, the rank order of *in vitro* activities of seven FQs against 140 clinical *Acinetobacter baumannii* isolates was in the following order: clinafloxacin > gatifloxacin > levofloxacin > trovafloxacin > gemifloxacin = moxifloxacin > ciprofloxacin *. Noteworthy, the inhibitory effects of ciprofloxacin against different Gram-positive and Gram-negative bacteria and a schematic view of this antibiotic's clinical usage are presented in Tables 1, 2, respectively.

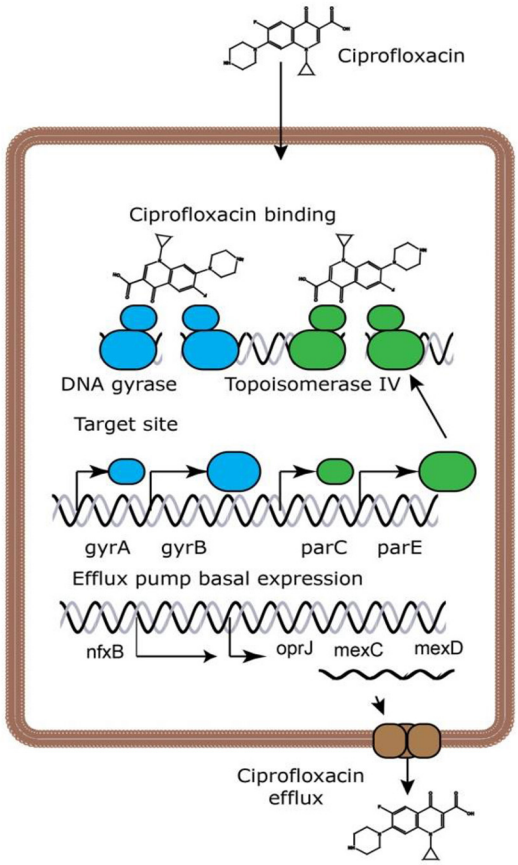


FIGURE 2
Ciprofloxacin's mechanisms of action (13). Because ciprofloxacin blocks DNA gyrase and topoisomerase IV, DNA replication is slowed and double-stranded DNA breaks are created.

Anti-biofim effects

Biofilm is made up of cell masses that are located in an environment in their extracellular matrix. This matrix contains polysaccharides, proteins, nucleic acids, and lipids. Biofilms are involved relatively in 80% of human infections (64). Biofilm is one of the most essential factors in developing tolerance against antimicrobial agents (65). Ciprofloxacin is an antibiotic agent that has the potential to control biofilm (66). Reffuveille et al. studied the anti-biofilm effect of ciprofloxacin on *E. coli* and *P. aeruginosa.* The percentage of biofilms remained in the primary biofilms after growth in a medium containing ciprofloxacin (320 ng/ml or 20 μM) + carboxy-TEMPO (4-carboxy-2, 2, 6, and 6- tetramethylpiperidine 1-oxyl) were 0.7 and 13% in *P. aeruginosa* PA14 and *E. coli* O157, respectively. However, using TEMPO without ciprofloxacin revealed that 40% of *P. aeruginosa* and 29% of *E. coli* remain. Hence, it can be concluded that the presence of ciprofloxacin is necessary for decreasing biofilm (67).

Verderosa et al. also evaluated the effect of ciprofloxacin-nitroxide and ciprofloxacin-methoxamine hybrids on *P. aeruginosa* PA14 biofilm at 20- and 40-μM concentrations. When using ciprofloxacin -nitroxide at 20 μM, 80% reduction was observed in the total biofilm; however, half of the biofilm biomass was composed of dead cells, which is suggestive of a 90% reduction in the live cell volume. The use of ciprofloxacin -methoxamine at 40 μg indicated a low reduction in biofilm bio-volume (41%). In addition, 91% of 59% of remaining biomass was composed of dead cells, corresponding to an overall reduction of 95% in live-cell volume, showing a 5% improvement compared to 20 μM. As a result, the higher doses of ciprofloxacin -nitroxide have more potential against *P. aeruginosa* but less capability in removing biofilm, which may be due to the release of cellular adhesive contents (such as DNA) into the environment. Ciprofloxacin-methoxamine reduce biofilm volume by 30% at 20 μM and by 35% at 40 μM concertation, which proves that it has less effect on biofilm than ciprofloxacin-nitroxide (68).

Therefore, recent studies have reported the antibiofilm effect for ciprofloxacin against Gram-negative bacteria. However, these data are limited, and the exact interaction of this antibiotic with bacterial biofilm is not reported. Therefore, future studies should be evaluated molecular and microscopic interactions of ciprofloxacin with the biofilm community of microorganisms;

TABLE 1 The inhibitory effect of ciprofloxacin against various Gram-positive and Gram-negative bacteria.

	Bacteria	MIC of ciprofloxacin	References
Gram-positive bacteria	*Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, and MRSA	MIC range 0.12–1 mg/L	(5)
	Streptococcus pneumoniae	MIC range 0.5–6.3 mg/L	
	Enterococcus faecalis	MIC range 0.5–6.3 mg/L	
	MRSA	0.1–0.8 mg/L	(14)
	Streptococcus pyogenes	0.5–1.6 mg/L	
	Enterococcus faecalis	0.8–25 mg/L	
	MRSA	1 mg/L	(8)
	S. pneumoniae and *E. faecalis*	2 mg/L	
	Streptococcus pyogenes	4 mg/L	
	Staphylococcus spp.	0.5 mg/L	(15)
	Enterococcus spp.		
	Streptococcus serogroups A	1 mg/L	
	MRSA S. pneumoniae	0.5 mg/L	(16)
	Enterococcus faecalis	2 mg/L	
	Mycobacterium tuberculosis	MIC range 0.5–1 mg/L	(17)
	Bacillus anthracis	0.03 mg/L	(18)
Gram-negative bacteria	*Escherichia coli*	MIC range 0.004–0.25 mg/L	(19)
		≤0.06 mg/L	(16)
		MIC range 0.01–2 μg/ml	(14)
		≤0.25 mg/L	(20)
		8–128 mg/L	(21)
		32–512 mg/L	(22)
	Klebsiella pneumoniae	≤0.06–0.125 μg/ml	(16)
		0.03 mg/L	(15)
		0.008–0.12 mg/L	(19)
		0.005–0.1 mg/L	(14)
	Proteus mirabilis	MIC <1 mg/L, and only 20% of the strains had MIC ≥1 mg/L	(23)
		MIC range ≤0.06–0.125 mg/L	(16)
		≤1 mg/L	(24)
		≤0.01–0.1 mg/L	(14)
	Haemophilus influenzae	0.008–0.015 mg/L	(19)
		≤0.01 mg/L	(14)
		MIC range 0.015–0.03 mg/L	(25)
	Moraxella catarrhalis	0.023–0.25 mg/L for β-lactamase-mediated isolates and 0.047–0.125 mg/L for non-β-lactamase-mediated isolates	(26)
		MIC range 0.002–2 mg/L	(27)
		MIC range 0.015–0.06 mg/L	(28)
	Legionella pneumophila	≤0.125 mg/L	(29)
		0.06 mg/L	(30)
		0.015–0.03 mg/L	(31)
	Neisseria meningitidis	≤0.01 mg/L	(14)
		0.006 mg/L	(32)
	Neisseria gonorrhoeae	0.015 mg/L	(33)
		0.008 mg/L	(34)
	Pseudomonas aeruginosa	0.5–8 mg/L (the susceptibility of *P. aeruginosa* to ciprofloxacin was 80%)	(35)

(Continued)

TABLE 1 (Continued)

Bacteria	MIC of ciprofloxacin	References
	1 mg/L (the percentage of susceptibility of *P. aeruginosa* was 90%)	(25)
	5 mg/L	(36)
	0.016 mg/L	(37)
Acinetobacter baumannii	≤0.03–>128 mg/L	(38)

MRSA, methicillin-resistant S. aureus; MIC, minimum inhibitory concentration.

additionally, the anti-biofilm activity of ciprofloxacin should be assessed against multi-species biofilm.

Resistance mechanisms against ciprofloxacin

Antibiotic resistance is one of the most severe public health issues facing the globe today. Antibiotic-resistant organisms can quickly spread, posing a hazard to populations in the form of novel infectious disease strains that are more difficult to cure and treat (69). Treatment failures may occur due to microbial resistance to effective broad-spectrum antibiotics. Treatment failures and difficult-to-treat infections could lead to a high death rate. Drug target mutations (DNA gyrase and DNA topoisomerase IV), mutations that limit drug accumulation, and plasmids that shield cells from ciprofloxacin's deadly effects are the three mechanisms of ciprofloxacin resistance that have been found (70).

Alterations in target enzymes

Ciprofloxacin resistance in topoisomerase IV or gyrase can result from a single amino acid change. The amino-terminal domains of *GyrA* (residues 67 to Tyr122 for GyrA, Tyr120 for *ParC*) or *ParC*, which are covalently bound to DNA in an enzyme intermediate (106 for *E. coli* numbering), are where these resistance mutations are most frequently detected (residues 63–102). They are near the tyrosine active site. This domain is referred to as the quinolone resistance determining region (QRDR) of *GyrA* and *ParC* (71).

Quinolone resistance has also been linked to changes in specific domains of *GyrB* and *ParE*; however, these alterations are far less common in resistant clinical bacterial isolates than mutations in *GyrA* or *ParC*. Ciprofloxacin resistance has increased with sequential mutations in both target enzymes. High-level quinolone resistance is frequently associated with mutations in gyrase and topoisomerase IV in several species (72).

Altered drug permeation

In Gram-positive bacteria, active efflux transporters are the main mechanism for reducing cytoplasmic drug concentrations. It has not been demonstrated that decreased diffusion through the cytoplasmic membrane is a form of resistance. Reduced outer membrane porin diffusion channels, which are necessary for ciprofloxacin to enter the periplasm, may be a factor in the development of resistance in Gram-negative bacteria and cooperate with basal or elevated expression of efflux transporters (72).

Porins are the main route for hydrophilic antibiotics like FQs to enter the bacterial outer membrane. Coexisting resistance mechanisms such as efflux pumps or antibiotic degrading enzymes are amplified by lower antibiotic uptake due to alterations in porin expression, resulting in high-level resistance (73).

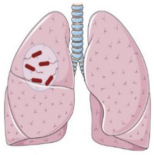
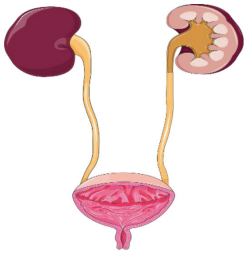
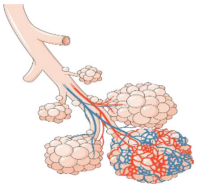
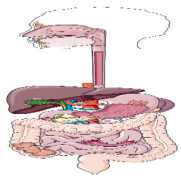
Plasmid-mediated quinolone resistance

Horizontal transference has been identified as the principal method for spreading quinolone resistance globally since 1998, when the primary plasmid-mediated quinolone resistance gene (PMQR) was first identified in a *K. pneumoniae* strain in the USA (74). The lowest inhibitory concentrations (MIC) of FQs, which typically prevent their *in vitro* detection, impart a modest growth in the presence of these resistance determinants. Furthermore, taking into account high-degree resistance to widen, PMQR might contribute to an increase in the occurrence of spontaneous mutations in QRDRs (72, 74, 75).

Inducing low susceptibility to these drugs by protecting the binding site in DNA-gyrase (*qnr* gene), modifying the drug enzymatically (*aac(6')-Ib-cr* gene), and expelling the agent from its site of action by coding for efflux pumps (*oqxAB* and *qepA* genes) are currently the three main mechanisms of resistance to quinolones related to PMQR that are recognized (74, 76).

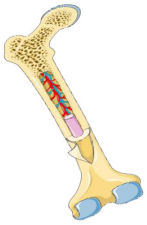
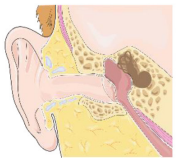
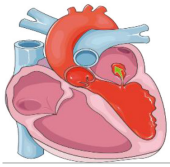
PMQR genes consist of six *qnr* genes (*qnrA, qnrB, qnrC, qnrD, qnrS,* and *qnrVC*) encoding gyrase-protection repetitive peptides *oqxAB*, *qepA*, and *qaqBIII* encoding efflux pumps (77, 78); and *aac(6′)-Ib-cr* encoding an aminoglycoside and quinolone inactivating acetyl-transferase (79). These genes

TABLE 2 Schematic view of clinical usage of ciprofloxacin.

Infection	Notes	References
Tuberculosis 	MIC of ciprofloxacin against Mtb ranges between 0.5 and 1 mg/L, and a single oral 750-mg dose of ciprofloxacin has been shown to produce a serum level of 2.01 mg/L, with a bronchial tissue level of 4.86 mg/kg	(39)
	Ciprofloxacin may be effective in treating Mtb, especially in patients with HIV infections and MDR-TB, in combination with other anti-mycobacterial drugs	(40)
	According to WHO consolidated guidelines on tuberculosis, ciprofloxacin is no longer recommended for treating drug-resistant Mtb	(41)
	Mtb sensitivity to ciprofloxacin can be decreased after short exposure courses, which makes this drug ineffective in treating Mtb	(42)
Gonorrhea 	The CDC declared that ciprofloxacin is no longer recommended for the treatment of gonorrhea	(43)
Malaria 	A combination of ciprofloxacin with rapidly acting antimalarial agents such as mefloquine can be a valuable treatment for resistant *Plasmodium falciparum* infections	(44)
UTI 	Ciprofloxacin is the most commonly prescribed FQ for the empirical treatment of UTIs because of its availability in oral and intravenous forms	(45, 46)
	Ciprofloxacin is effective for treating acute uncomplicated cystitis in 3-day regimens. However, having a propensity for side effects suggests using ciprofloxacin for more important diseases and considers it an alternative drug for acute cystitis	IDSA guideline (2010 update)
	For treating acute pyelonephritis, an oral 500-mg dose of ciprofloxacin twice a day for seven days with or without an intravenous 400-mg of ciprofloxacin is recommended in regions with <10% of uropathogens resistance	(47)
	Considering the adverse reactions of ciprofloxacin, FDA has recommended not to use ciprofloxacin for uncomplicated UTIs when other choices are available	(48)
	During the last decade, the resistance of uropathogens against ciprofloxacin has increased. In a 10-year follow-up of *E. coli*, a significant increase in ciprofloxacin resistance from 1.8 to 15.9% was observed	(49)
	Ciprofloxacin ER, a once-daily formulation with delayed release, achieves a higher Cmax and has more rapid bacterial killing, which makes it a valuable option for treating out-patient UTIs	(50)
Respiratory tract infections 	Ciprofloxacin is effective against the most frequent bacterial respiratory pathogens such as *H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, and *P. aeruginosa* and can be used to treat complicated and severe lower respiratory tract infections	(51)
	Ciprofloxacin can be used for treating pneumonia (mainly nosocomial), and chronic bronchitis, as well as CF	(51)
	A combination of oral ciprofloxacin with a nebulized antibiotic* (is suggested as first-line therapy), and a 2-week treatment of ciprofloxacin for CF patients who are chronically infected with *P. aeruginosa* is recommended	(52)
	Ciprofloxacin dry powder inhaler was developed for targeted lung delivery, which achieves a high concentration of ciprofloxacin in the lungs with low systemic exposure	(51, 53)
Gastrointestinal infections 	Ciprofloxacin has great mucosal tissue levels and low MICs against *Helicobacter pylori*, but it has failed to eradicate this bacterium because of reduced antibiotic activity in a low pH environment and increased ionization and gastric mucus trapping of ciprofloxacin	(54)
	Ciprofloxacin is the critical choice for treating adult patients with typhoidal and severe non-typhoidal salmonellosis with spreading infection beyond the intestinal tract	(55)

(Continued)

TABLE 2 (Continued)

Infection	Notes	References
Bone, joint, and soft-tissue infections 	Administration of an oral 750–1,000 mg dose of ciprofloxacin every 12 h can cure most cases of Gram-negative osteomyelitis or mixed infections with *S. aureus*	(56) (57)
	Concerning the increasing rate of resistance against ciprofloxacin, this antibiotic should not be used for the treatment of simple SSTIs but should be reserved for patients with allergies to β-lactams	
Ear infections 	Topical ciprofloxacin has the advantages of direct contact with infected tissue, excellent empiric coverage, non-ototoxicity, and no risk of musculoskeletal complications, which often are associated with systemic use	(58)
	Overuse has increased the prevalence of ciprofloxacin-resistant otologic infections in recent years, which can cause serious challenges in treating ear infections due to the limited options for topical therapy	(59)
	The results of a study indicated that ciprofloxacin was ineffective for treating ciprofloxacin-resistant infections, and other alternatives should be explored	(59)
	The results showed that ciprofloxacin was the most effective antibiotic for the treatment of CSOM, with 93.7% sensitivity of *P. aeruginosa* isolates and high susceptibility rates in Staphylococci, *Klebsiella*, and *Proteus* spp	(60)
Endocarditis 	Oral ciprofloxacin is effective against *Pasteurella multocida*, *Neisseria*, and the HACEK group. It causes endocarditis and can be used in patients with low tolerance to β-lactams	(61, 62) (63)
	A combination of oral ciprofloxacin and rifampicin successfully treats right-sided Staphylococcal endocarditis; however, increasing resistance to these agents is a concern	

CDC, Centers for Disease Control and Prevention; UTI, urinary tract infection; ER, extended-release; FQ, fluoroquinolones; IDSA, Infectious Diseases Society of America; CF, cystic fibrosis; MIC, minimum inhibitory concentrations; SSTIs, skin and soft tissue infections; Mtb, Mycobacterium tuberculosis; CSOM, chronic suppurative otitis media.
*Mainly inhaled colistimethate sodium.

can synergize with chromosomal *gyrA* and *parA* mutations, increase the mutant prevention concentration of quinolones, interfere with quinolone action in apparently susceptible bacteria harboring them (80), and confer evolutionary fitness unrelated to quinolone resistance (Figure 3) (81).

In some *Enterobacteriaceae* species, the co-existence of mutations in the QRDR and PMQR genes can may occur. Additionally, QRDR mutations that increase FQs resistance can be encouraged by the presence of PMQR determinants (83). According to the findings of the Egyptian study, a high level of resistance to FQs is conferred by the accumulation of PMQR genes and QRDR mutations (84).

Mechanism of resistance in gram-negative bacteria

Neisseria

Single nucleotide polymorphisms (SNPs) in *gyrA* alone confer low- to intermediate-level resistance in *N. gonorrhea*, whereas high-level resistance necessitates one or more specific concurrent mutations in *parC*. These changes can be easily selected and transferred to other gonococci by exposing them to sub-inhibitory ciprofloxacin doses (85).

A missense mutation in *gyrA* (S91F) within the QRDR has been demonstrated to cause a 100-fold increase in ciprofloxacin resistance. A subsequent mutation at codon 95 (D95N) resulted in a two-fold increase in ciprofloxacin resistance. Higher levels of quinolone resistance required mutations in *parC* in addition to those in *gyrA*. These *parC* mutations were found in codons 88 (S88P) and 91 (E91K) of the *parC* gene (85). Additional GyrA/ParC amino acid change patterns were later discovered in ciprofloxacin-resistant bacteria worldwide (86). Ciprofloxacin resistance in Gonorrhea appears unaffected by mutations in the *gyrB* and *parE* genes (86).

As mentioned, the most common combinations of amino acid substitutions in the GyrA and ParC proteins conditioning resistance to FQs are S91F + D95G/A in GyrA and S87R in ParC (87). This combination was found in more than 40% of *N. gonorrhoeae* strains resistant to FQs and conditioned the MIC of ciprofloxacin from 4 to 32 mg/L (87). The frequency of individual mutations in the *gyrA* and *parC* genes varies (88). A mechanism increasing FQ MIC values, based on the overproduction of NorM membrane pump proteins, was also described in single *N. gonorrhoeae* strains (86–89).

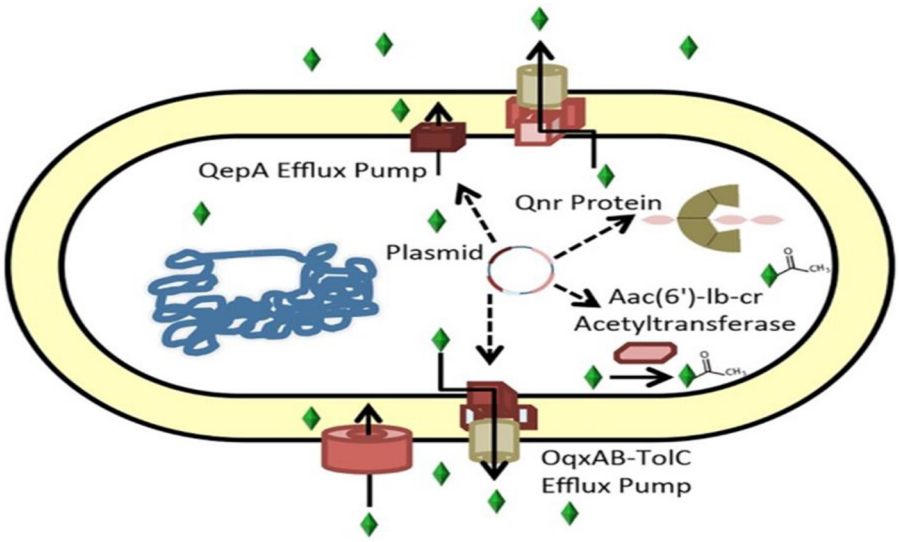


FIGURE 3
Mechanisms of ciprofloxacin resistance carried by plasmids (82). Genes encoding the ciprofloxacin efflux pumps can be found in plasmids. Aac(6')-Ib-cr, an aminoglycoside-modifying acetyltransferase that acetylates and inactivates ciprofloxacin, or QepA or OqxAB, the Qnr protein, which binds gyrase.

In gonococci, four efflux pump systems (MtrCDE, MacAB, NorM, and FarAB) have been discovered in most strains. The MtrCDE, MacAB, NorM, and FarAB systems belong to the RND, ABC, MATE, and MF families, respectively, which have been proven to identify antimicrobials previously or currently approved for gonorrhea treatment (89, 90).

The MICs for ciprofloxacin-resistant *N. meningitides* isolates have been reported to range between 0.06 and 0.25 g/ml, with mutations in the QRDR of the gyrase-encoding gene *gyrA* being responsible for the majority of this resistance (91–93). According to a Chinese study, mutations in the QRDR of *gyrA* related with quinolone resistance substitutions were observed in all of the 51 ciprofloxacin-non susceptible *N. meningitides* strains, of which 49 strains harbored the typical substitution of threonine to isoleucine at amino acid position 91 (T91I). The other two ciprofloxacin-intermediate strains, harbored aspartate to asparagine substitutions at amino acid position 95 (D95N). No additional mutations were observed in the QRDRs of *gyrB, parC,* or *parE*. Furthermore, sixteen *gyrA* alleles (R1–R16), were defined in 51 ciprofloxacin-non susceptible isolates. Most of ciprofloxacin resistance-conferring alleles were transmitted through horizontal gene transfer (93).

The *gyrA* gene of *N. meningitidis* is 95% identical to the *gyrA* gene of *N. gonorrhoeae.* Mutations in the *gyrA* gene have been associated with ciprofloxacin resistance in *N. meningitidis.* The QRDR from the resistant *N. meningitidis* contained a mutation that resulted in an Asp95-to-Asn change. This known change in the *N. gonorrhoeae gyrA* gene's QRDR raises ciprofloxacin MICs to levels similar to those seen in this strain (94).

In the QRDR of *gyrA*, nearly all previously identified ciprofloxacin-resistant *N. meningitidis* isolates had Ile (I) or Phe (F) mutations at position 91 (92, 95–97). In *N. meningitidis*, further mutations in *gyrA* (D95N and T193A), as well as *parC* (D86N, S87R, and E91G), have been linked to increased ciprofloxacin MICs (92, 93, 97, 98). Chen et al. found that all quinolone-resistant *N. meningitidis* isolates contained mutations in T91 and/or D95 of GyrA, with seven isolates also possessing ParC mutations and displaying higher MICs. The specific *Neisseria lactamica* donors of seven mutation-carrying *gyrA* alleles (*gyrA*92, *gyrA*97, *gyrA*98, *gyrA*114, *gyrA*116, *gyrA*151, and *gyrA*230) and the *Neisseria subflava* donor isolate of *gyrA*171 were discovered by genomic analysis. Transformation of *gyrA* fragments from these donor strains into a meningococcal isolate raised its ciprofloxacin MIC from 0.004 g/ml to 0.125 or 0.19 g/ml and to 0.5 g/ml with the further transformation of an additional ParC mutation, according to their findings. Over fifty percent of quinolone-resistant *N. meningitides* strains acquired resistance through horizontal gene transfer from three commensal *Neisseria* species (97).

According to a study conducted in Brazil, all of the ciprofloxacin -resistant *N. meningitides* isolates possessed a Thr to Ile mutation at the QRDR of the *gyrA* gene's amino acid 91. No further mutations were identified in the *gyrA* or *parC* QRDRs (91). According to a study in Spain, single mutations in the *gyrA* (with Thr-91 to Ile being the most common substitution found) of ciprofloxacin-resistant *N. meningitidis* were the primary mechanism implicated. Four distinct *gyrA* substitutions were found in two meningococci. There were no changes in the *parC*

and *gyrB* genes' QRDRs. However, three strains had a His-495 to Asn substitution in the *parE* gene. In addition, two distinct mutations in the *mtrR* gene that impact the expression of the MtrCDE efflux mechanism were discovered (99).

Pseudomonas aeruginosa

Two basic pathways of ciprofloxacin resistance in *P. aeruginosa* have been thoroughly explored. Major contributors to ciprofloxacin resistance in *P. aeruginosa* are mutations in the ciprofloxacin target-encoding genes *gyrAB* and *parCE* that decrease the affinity of DNA gyrase or topoisomerase for ciprofloxacin (13). Furthermore, overexpression of efflux pumps to lower antibiotic intracellular concentrations promotes ciprofloxacin expulsion from *P. aeruginosa* cells due to mutations in efflux pump regulatory genes. It has become apparent that a wide number of additional genes can play a role in ciprofloxacin resistance and that resistance evolves through a mix of alleles, underscoring the multifactorial character of the ciprofloxacin resistance development process (13). Bacteria with both target-site mutations and efflux overexpression were more resistant to ciprofloxacin than bacteria with only individual mutations (100).

Sequence variants in which the Thr at position 83 in GyrA is replaced by Ile and the Ser at position 87 in ParC is replaced by a Leu are the most commonly occurring alterations associated with ciprofloxacin resistance in *P. aeruginosa* isolates from patients and *in vitro* evolved isolates (100).

The second most common GyrA variation occurs at position 87, where Asn, Tyr, or Gly residues replace aspartate (13). The presence of alternative amino acid residues at these positions decreases gyrase's affinity for ciprofloxacin, providing a molecular explanation for the GyrA variations' increased ciprofloxacin resistance (101). GyrA and ParC variants are more common than GyrB and ParE variants, possibly because alterations in GyrB, and ParE sequences give lower-level ciprofloxacin resistance. In clinical isolates of *P. aeruginosa*, resistance alleles in both *gyrA* and *parC* give stronger ciprofloxacin resistance than resistance alleles in only *gyrA* (102–104).

Four efflux pumps in *P. aeruginosa* are known to efflux FQs: MexCD-OprJ, MexEF-OprN, MexAB-OprM, and MexXY-OprM (105–107). System-specific regulatory proteins regulate efflux pump gene expression, and mutations in these regulators cause efflux pump overexpression (108, 109). Two efflux pumps are overexpressed. Most typically, MexCD-OprJ and MexEF-oprN have been implicated with ciprofloxacin resistance. Overexpression of MexCD-OprJ occurs in *P. aeruginosa* isolates from Cystic Fibrosis (CF) and non-CF patients and is caused by mutations in the *nfxB* gene (110, 111).

Overexpression of MexEF-OprN occurs in isolates of *P. aeruginosa* from CF and non-CF patients due to mutations in the *mexS* gene, which result in overexpression of MexT. The MexEFoprN genes are regulated by the transcription factor MexT (106).

Furthermore, overexpression of MexXY-OprM in clinical isolates of *P. aeruginosa* has been demonstrated to confer ciprofloxacin resistance at lower levels. Mutations in the regulator gene *mexZ* have been blamed for most MexXY-OprM overexpression (100). Experiments demonstrate that a *gyrA*-resistant allele mutation is required for ciprofloxacin resistance, with other mutations enhancing resistance further (112). GyrA's great affinity for ciprofloxacin makes it possible for bacteria to harbor mutations in the regulatory genes of efflux pumps, yet a wild-type *gyrA* allele may still be vulnerable to the drug (100).

Campylobacter

The most prevalent mechanism of ciprofloxacin resistance in *Campylobacter* is a single point mutation C257T in the *gyrA* gene, located within the QRDR resistance (113). This causes a Thr to Ile amino acid change in the Gyrase A subunit at position 86 (114). Other mutations in the *gryA* gene have been linked to increased ciprofloxacin resistance but at lower doses and frequencies (115–117). In *Campylobacter* spp., polymorphisms in the *gyrB* gene have been ruled out as a cause of quinolone resistance. The *gyrA* mutation interacts with the most frequent *Campylobacter* drug efflux pump, CmeABC, to promote the development of ciprofloxacin-resistant bacteria when its expression is raised (116, 117). Overexpression of the CmeABC efflux pump does not result in ciprofloxacin resistance without the *gyrA* gene mutation (114, 117, 118).

The 16-bp inverted repeat (IR) in the *cmeR-cmeABC* intergenic region is one more element that heightens resistance. The percentage of resistant isolates increases and the average ciprofloxacin MIC increases when this mutation coexists with the *C257T-gyrA* mutation. Recently found and spreading, *RE-cmeABC* is a variant of the *cmeABC* gene that increases ciprofloxacin resistance (118). Ciprofloxacin resistance may be indirectly impacted by changes in other genes. Variations in the mutant frequency decline gene (*mfd*), for instance, may be involved because silencing of this gene has been shown to 100-fold reduce mutation rates (117).

Haemophilus influenzae

Ciprofloxacin resistance in *H. influenzae* is associated to chromosome-mediated mutations in the QRDRs of the genes producing DNA gyrase and topoisomerase IV, including *gyrA, gyrB, parC,* and *parE*. *GyrA* (at Ser84 and Asp88) and *parC* (at Gly82, Ser84, and Glu88) had more amino acid changes than *gyrB* and *parE* (119). Puig et al. found that strains with a single

alteration in GyrA or one change in GyrA plus one in ParC had ciprofloxacin MICs of 0.12 to 2 g/ml.

In contrast, those with three or four changes (in GyrA, ParC, and ParE) had higher MICs (8–16 g/ml) (120). Ser84 to Leu or Tyr and Asp88 to Tyr, Asn, or Gly were the most common alterations in GyrA, which have been linked to resistance in *H. influenza* (119). In ParC, the most common changes were Ser84Ile and Glu88Lys (119) and Ser84Arg (119).

Enterobacteriaceae

Ciprofloxacin resistance in *Enterobacteriaceae* has been extensively researched (121, 122). The accumulation of mutations in the genes encoding the two quinolone targets: DNA gyrase and topoisomerase IV in *E. coli* is a major contributor to resistance and decreased sensitivity to quinolones (123).

It may just take one change to the *E. coli* gene *gyrA* to result in large levels of nalidixic acid resistance. However, additional, progressive mutations in the topoisomerase IV or *gyrA* genes are necessary for high-level FQs resistance, including ciprofloxacin. *Escherichia coli* was the only species in a study of eight *Enterobacteriaceae* species where multiple mutations in *gyrA* were necessary for high-level FQ resistance. The most frequent *gyrA* mutations identified in clinical, veterinary, and laboratory strains of *E. coli* occur at codon 83. This Ser residue is most frequently changed to Leu in *E. coli* isolates with high levels of nalidixic acid resistance and lower susceptibility to FQs*.

Strains with a somewhat higher resistance to FQs had an extra mutation, most frequently at codon Asp87. The high occurrence of mutations at Ser83, however, has a plausible explanation because strains with a single mutation at Ser83 were considerably more resistant to FQs than those with a single mutation at Asp87*.

Increased drug extrusion caused by overexpression of AcrAB-TolC, the principal efflux pump reported in *Enterobacteriaceae*, on the other hand, is a major source of worry because it confers cross-resistance to a variety of unrelated chemicals, including antimicrobials. Other efflux systems, such as AcrEF and EmrAB, have been reported to engage in the extrusion of antimicrobial compounds to a lesser amount (124).

Increased efflux has been identified as the main mechanism for the development of quinolone resistance in *Salmonella*. On the other hand, in these bacteria, decreased OmpF porin synthesis has occasionally been linked to the MDR phenotype. Furthermore, according to a study, the ParC T57S substitution was common in strains exhibiting the lowest MICs of ciprofloxacin, while increased MICs depended on the type of GyrA mutation. PMQR genes represented a route for resistance development without target-site mutations (125).

According to Azargun et al., high-level ciprofloxacin resistance in *Enterobacteriaceae* is linked to DNA gyrase and topoisomerase IV mutations as a primary mechanism and PMQR genes *acrB* efflux pump gene expression, and outer membrane *ompF* gene expression. Ciprofloxacin resistance is increased due to twin mutations in *gyrA* and *parC* (124). PMQR genes are not the critical mechanism of ciprofloxacin resistance in uropathogenic *E.coli* in South Iran, according to Malekzadegan et al. (126).

Legionella pneumophila

Legionella pneumophila resistance to ciprofloxacin is most typically linked to changes in *gyrA*, *gyrB*, *parC,* and *parE* genes. Mutations affecting codons 83 and 87 of the *gyrA* QRDR have been linked to the *in vitro* selection of *L. pneumophila* strains with high-level ciprofloxacin resistance (127). However, mutations in *gyrB* and *parC* have also been identified (127). *In vivo*, only mutations at codon 83 of the *gyrA* gene have been described (128, 129).

Using next-generation DNA sequencing (NGS), Shadoud et al. demonstrated that the 248CT (T83I) mutation-carrying L. pneumophila mutant population was rapidly selected *in vivo* in two legionellosis patients treated with ciprofloxacin, increasing from 1.05% of the total *L. pneumophila* lung population at the time of diagnosis to 94% after a few days of FQ treatment (129).

Moraxella catarrhalis

According to a study, an amino acid substitution of Thr80 to Ile in GyrA causes *M. catarrhalis* to have low-level resistance to FQs (130). FQ targets gyr and par were also sequenced in another work on isolates with decreased FQ resistance that were produced by stepwise selection in levofloxacin. GyrA (D84Y, T594dup, and A722dup), GyrB (E479K and D439N), and ParE (Q395R) were shown to have six new mutations that contribute to *M. catarrhalis* resistance to FQs (131). According to a Polish study, *M. catarrhalis* FQ resistance is linked to amino acid changes in the *gyrA* and *gyrB* genes. G412C and four silent transition mutations were found in the *gyrA* gene. Two identical silent mutations and the substitution A1481G occurred in the *gyrB* gene (132).

Acinetobacter baumannii

Resistance to ciprofloxacin in *Acinetobacter baumannii* is advanced *via* unique techniques, one of which is the modifications that took place within the expression of the efflux pumps. The efflux pump in *A. baumannii* is the AdeABC pump and is of great significance in phrases of resistance advent (133).

This efflux pump has a three-part structure and is a member of the resistance-nodulation-cell department (RND) family:

AdeB is a multidrug transporter, AdeC is an outer membrane protein, and AdeA is a membrane fusion protein. AdeS is a sensor kinase, while AdeR is a response regulator. Together, they form the -component system (AdeR-AdeS) that tightly controls the adeABC operon. Both point mutations in AdeRS and the insertion sequence (IS) Aba-1 insertion upstream of the adeABC operon have been implicated in the overexpression of the AdeABC efflux pump*.

The presence of quinolone resistance (*qnr*) genes on the plasmid, which results in a low-level resistance to quinolones, is another mechanism that results in resistance to ciprofloxacin (134). A mutation in the quinolone resistance-determining regions (QRDR), which affects the target enzymes of DNA gyrase (*gyrA*) and topoisomerase IV (*parC*), is another important mechanism. The main effects of quinolones are on target enzymes like DNA gyrase, which block the transcription process by attaching to and mutating this enzyme's gene (135).

Sequencing results in several studies revealed a serine to leucine mutation at position 83 of the *gyrA* subunit, indicating that Ser83Leu substitution is the primary mutation in *A. baumannii* for FQ resistance (136). In ciprofloxacin-resistant isolates from a different investigation, Ala84Pro or Gly81Val mutations in the *gyrA* gene were found. Quinolones' target in *A. baumannii* is topoisomerase IV, and mutations at *parC* residues Ser80 and Glu84 contribute to decreased fluoroquinolone sensitivity*.

Two clinical isolates from another study had mutations in *parC* without *gyrA*, suggesting that *parC* might not only be a secondary goal for quinolones but is as critical as *gyrA* to purpose a decreased susceptibility to FQs in *A. baumannii* (136). ParC mutations are typically in conjunction with mutations in *gyrA* and are needed to gather a high degree resistance to quinolones. *Acinetobacter baumannii* can resist FQs with just a single point mutation in DNA gyrase, but concurrent mutations in the QRDR regions of the *gyrA* and *parC* genes are projected to significantly contribute to high-degree FQs resistance (136).

A study found that the Serine 83 to Leucine mutation was present in the DNA gyrase subunit A's QRDR in isolates that were resistant to ciprofloxacin (GyrA). Furthermore, among isolates that were resistant to ciprofloxacin, researchers were unable to detect ParC mutations or plasmid-mediated quinolone resistance (qnrA). They came to the conclusion that a mutation in GyrA, with the presence of efflux pumps serving as a secondary motive, is the primary source of ciprofloxacin resistance in *A. baumannii* isolates from burn infections (137).

According other study in Iran, the prevalence rates of *qnrA, qnrB, qnrS, AdeA, AdeB*, and *AdeC* genes among *A. baumannii* isolates have been 0, 0, 3.9, 100, 100, and 100%, respectively. In all of the resistant isolates, mutation within the *gyrA* gene became discovered, however, no mutation became visible within the *parC* gene (138).

Mechanism of resistance in gram-positive bacteria

Enterococci

In *Enterococci*, ciprofloxacin resistance is mostly caused by chromosomal mutations in the genes encoding quinolone targets, DNA gyrase, and topoisomerase IV, which are mostly found in the QRDR (139). Resistance-associated mutations have been discovered in the *gyrA* gene (Ser83Arg, Ile, or Asn; Glu87Lys, Gly) and the parC gene in *E. faecalis* (Ser80Arg, or Ile; Glu84Ala). In *Enterococcus faecium*, mutations in the *gyrA* gene (Ser83Ala, Leu, Ile, Tyr, or Arg; Glu87Leu, Gly, or Lys) and the *parC* gene (Ser83Ala, Leu, Ile, Tyr, or Arg) have been identified (140).

Another well-known mechanism of quinolone resistance is antibiotic externalization *via* efflux pumps. NorA is described in *E. faecium* and EmeA in *E. faecalis* (141). A third resistance mechanism reported in *E. faecalis* is qnr, a protein with a series of pentapeptide repeats identical to the plasmid-borne quinolone resistance genes identified in *Enterobacteriaceae*. This protein protects DNA gyrase by preventing ciprofloxacin from binding to DNA and forming an antibiotic–gyrase complex (142).

Staphylococcus aureus

It has been well established over the past few decades that the pathogen's capacity for resistance to antimicrobial drugs, particularly methicillin, may contribute to its persistence in the hospital and community (143). Methicillin-resistant *S. aureus* (MRSA) are a global health concern due to their growing resistance to macrolide, lincosamide, and streptogramin B treatments (144). In clinical isolates of *S. aureus*, resistance to ciprofloxacin is caused by both mutations in topoisomerases that impair drug binding effectiveness and increased production of endogenous efflux pumps (72). Amino acid substitutions in residues that make up the drug-binding site, also known as the quinolone resistance-determining area, are the most common types of mutations (72). ParC is the topoisomerase with the highest sensitivity in *Staphylococci* and is thus the major target. The secondary target is DNA gyrase, which is less sensitive. FQs are highly effective for *Staphylococci*; thus, alterations in both enzymes are required to build a resistance that exceeds the MIC breakpoint. A single amino acid substitution will often increase the MIC by 8–16 times (145, 146).

Clinical isolates with strong ciprofloxacin resistance frequently overexpress chromosomally encoded efflux pumps. NorA is responsible for the ciprofloxacin and norfloxacin resistant, while NorB and NorC are responsible for the sparfloxacin and moxifloxacin resistant. Therefore, the overexpression of an efflux pump (NorA) leads to the ciprofloxacin resistance in *S. aureus* (72).

After challenging 222 isolates of *S. aureus* with the antibiotic ciprofloxacin, Papkou et al. (147) discovered that a single efflux pump, *norA*, causes widespread variation in evaluability across isolates, and that chemical inhibition of NorA effectively prevents resistance evolution in all isolates. The frequency of efflux pump genes driving ciprofloxacin and antiseptic resistance in MRSA isolates was studied in a study conducted in Iran. According to their findings, the *mdeA* and *qacA/B* genes were detected with the highest (61.7%) and lowest (3.3%) frequency, respectively, among ciprofloxacin-resistant isolates (148).

Mycobacterium tuberculosis

The second-leading cause of death worldwide among infectious diseases is TB, an old infectious disease caused by *M. tuberculosis* and other species that are closely related to it. Each year, an estimated two–three million people die from TB and its associated complications worldwide (149). DNA gyrase mutations, drug efflux pumps, bacterial cell wall thickness, and pentapeptide proteins (MfpA)-mediated gyrase regulation in *M. tuberculosis* are the ciprofloxacin -resistant mechanism in Mtb (150). Because mycobacteria lack topoisomerase IV, ciprofloxacin resistance mutations are found in the genes encoding gyrase, most commonly in the QRDR of *gyrA*, but sometimes in the QRDR of *gyrB*. Mutations mainly cause FQ resistance in tuberculosis in the *gyrA* gene, the most prevalent at locations 90, 91, and 94, which are associated with high-level resistance (151).

The most prevalent mutations in *gyrA* are found in the QRDR codons 88–94, particularly codons 88, 90, 91, and 94. FQ resistance in *gyrB* is often linked to mutations in codons 500 and 538 (152).

The incidence of *gyrA* mutations does, however, vary geographically. A study's mutational examination of samples from pulmonary TB patients revealed that the majority of the mutations change codons 94 (changing Asp with Gly, D94G), and 90 (replacing Ala with Val A90V). In MDR and treatment failure instances, the D94G mutation was most frequently linked to resistance to FQs. However, many A90V mutations were discovered in recently diagnosed patients (153).

The Mmr efflux transporter is the only efflux pump from the small multidrug resistance (SMR) family in the Mtb genome. It has been linked to *M. tuberculosis* resistance to dyes and antibiotics such as FQs (154). In a systematic assessment of *gyr* mutations, 64% of FQ-resistant *M. tuberculosis* isolates contained mutations in the QRDR of *gyrA*. In 534 resistant isolates, the QRDR of *gyrB* was sequenced, but only 3% exhibited mutations. Eighty-one percent of the *gyrA* mutations were found inside the QRDR, whereas 19% were found outside. In 54% of FQ resistant isolates, mutations in *gyrA* codons 90, 91, and 94 were found (substitutions at amino acid 94 accounted for 37%). Only 44% of the *gyrB* mutations were found inside the QRDR (155). Two amino acid positions, 74 and 88, are related to less prevalent genetic variants in *gyrA* (156).

Multiple mutations and codons 94, 90, and 88 of *gyrA* provided high-level FQ resistance (157). The considerably less common *gyrB* mutations (up to 10%−15%) were generally, but not consistently, associated with lower levels of FQ resistance (155). Nevertheless, combined *gyrA* and *gyrB* mutations could result in a substantially higher resistance level (155, 158). Other efflux pumps that may be involved in FQ resistance include antiporters LfrA and Tap, in addition to the mycobacterial pentapeptide MfpA and the ATPase complex Rv2686c-Rv2687c-Rv2688c operon (159). According to a study, ciprofloxacin-resistant clinical isolates of *M. tuberculosis* had significant efflux pump pstB transcripts in a few isolates, implying that the pump plays a role in resistance (160).

It should be noted that all of the mentioned resistance mechanisms are summarized in Table 3.

The combine use of ciprofloxacin with different antibacterial agents

Synergism of ciprofloxacin with aminoglycoside

Synergism of ciprofloxacin and amikacin for *P. aeruginosa* is as follows: total synergism–ciprofloxacin ¼ MIC + amikacin ¼ MIC, partite synergism–ciprofloxacin ½ MIC + amikacin $^{1}/_{16}$ MIC or ciprofloxacin $1/_{16}$ MIC + amikacin ½ MIC. Time-kill assay affirmed the synergistic activity of ciprofloxacin and amikacin, apparent at as early as 4 h and kept up after that*. Combining gentamicin with ciprofloxacin against *E. coli* and *P. aeruginosa* displayed the ideal treatment alternative. However, more *in vivo* and clinical trials are needed to determine the potential treatment regimen based on the combination of these two antibiotics (161).

Additionally, unlike when each antibiotic was used alone, combining tobramycin with either azithromycin or ciprofloxacin enhanced the killing of planktonic *K. pneumoniae* cells and accelerated bacterial clearance in a mouse model of cutaneous abscess infection. Additionally, combining ciprofloxacin and tobramycin increased the bactericidal activity against cells linked to biofilms. In this regard, the antibiotic combinations reduced the number of bacteria from 108 to fewer than 10 colony forming units (CFU) ml^{-1}; however, when each antibiotic was used alone, only 500 CFU ml^{-1} of bacteria were recovered (162).

In view of these findings, ciprofloxacin and tobramycin may be used in combination to treat both acute and persistent *K. pneumoniae* infections. The use of the aforementioned combination therapy can also lessen the emergence of resistance to individual or groups of antibiotics.

TABLE 3 Mechanisms of ciprofloxacin resistance in different bacteria.

Bacteria	Mechanism of resistance	References
Neisseria gonorrhea	1. Target-site modification (gyrA SNPs: S91F, D95N, and D95G, in the QRDR and parC SNPs: D86N, S88P, and E91K, in the QRDR) 2. An overexpressed NorM efflux pump	(85, 86, 89)
Neisseria meningitidis	1. Mutations in the QRDR of the gyrase-encoding gene gyrA [Ile (I) or Phe (F) mutations at position 91] ✓ Further mutations in *gyrA* (D95N and T193A) and *parC* (D86N, S87R, and E91G) have been linked to increased ciprofloxacin MIC 2. The Over expression of the MtrCDE efflux mechanism (by two distinct mutations in the mtrR gene)	(92, 93, 95, 97–99)
Pseudomonas aeruginosa	1. Target-site modification (Most common: replacement of Thr at position 83 in GyrA is by Ile and the Ser at position 87 in ParC by a Leu) 2. Efflux overexpression (MexCD-OprJ, MexEF-OprN, MexAB-OprM, and MexXY-OprM)	(13, 100, 105, 107)
Campylobacter jejune	1. Single point mutation C257T in the gyrA gene 2. Overexpression of efflux pump CmeABC 3. Inverted repeat (IR) in the cmeR–cmeABC intergenic region	(113, 114, 118)
Haemophilus influenza	1. Amino acid changes in the QRDR of the topoisomerase II and I genes ✓ gyrA (Ser84 and Asp88) and parC (Gly82, Ser84, and Glu88) had more amino acid changes than gyrB and pare	(119)
Escherichia coli	1. Mutations in the DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) are a major contributor to resistance [*gyrA* mutations (Nucleotide substitutions at codon 83)] and additional mutation, most commonly at codon Asp87 2. Overexpression of AcrAB-TolC, (the principal efflux pump)and AcrEF and EmrAB 3. Decreased expression of OmpF	(123, 124)
Salmonella	1. Increased efflux (a primary mechanism) 2. Decreased production of the OmpF porin 3. Mutations in gyrA and parC	(125, 126)
Legionella	1. Mutation in the gyrA/gyrB and parC/paeE (mostly mutations affecting codons 83 and 87 of the gyrA QRDR)	(127)
Moraxella catarrhalis	Amino acid substitutions in gyrA and gyrB gene (Amino acid substitution of Thr80 to Ile in GyrA: low-level resistance)	(130)
Acinetobacter	1. Expression of the efflux pumps. (AdeABC pump) 2. Presence of quinolone resistance (*qnr*) genes located on the plasmid, (low-level resistance) 3. Mutation in quinolone resistance-determining regions (QRDR), where the target enzymes of DNA gyrase (*gyrA*) and Topoisomerase IV (*parC*)	(133) (138) (135)
Enterococci	1. Chromosomal mutations in gyrA and parC ✓ Resistance-associated mutations have been discovered in the *gyrA* gene (Ser83Arg, Ile, or Asn; Glu87Lys, Gly) and the parC gene in *E. faecalis* (Ser80Arg, or Ile; Glu84Ala) ✓ In *E. faecium*, mutations in the gyrA gene (Ser83Ala, Leu, Ile, Tyr, or Arg; Glu87Leu, Gly, or Lys) and the parC gene (Ser83Ala, Leu, Ile, Tyr, or Arg) have been identified 2. Overexpression of active efflux (NorA in *E. faecium* and EmeA in *E. faecalis*) 3. Target protection (Qnr-like determinants), Binds gyrase, described in *E. faecalis*	(139, 140, 142)
Staphylococcus aureus	1. Mutations in the QRDR of DNA gyrase and topoisomerase IV ✓ ParC is major target. The secondary target is DNA gyrase, which is less sensitive 2. Overexpression of the efflux pump NorA	(145, 147)
Mycobacterium tuberculosis	1. Mutations in the genes encoding gyrase, most commonly in the QRDR of gyrA, but sometimes in the QRDR of gyrB ✓ The most prevalent mutations in gyrA are found in the QRDR codons 88–94, particularly codons 88, 90, 91, and 94 ✓ FQ resistance in gyrB is often linked to mutations in codons 500 and 538 2. Overexpression of The Mmr efflux pump and other efflux pumps include antiporters LfrA and Tap, in addition to the mycobacterial pentapeptide MfpA and the ATPase complex Rv2686c-Rv2687c-Rv2688c operon	(152, 154, 159)

CIP, ciprofloxacin; QRDR, quinolone resistance determining region; MIC, minimum inhibitory concentration.

Finally, the results of another study indicated that the outcomes of ciprofloxacin -streptomycin combination with cefotaxime represented a synergic impact on MDR *P. aeruginosa* and a noteworthy lessening in the MIC value at a ratio of 1:3 for 20 strains with the percentage of 95.23% (163). Thus, synergistic results of cefotaxime or streptomycin– ciprofloxacin make this combination beneficial. However, more studies of *P. aeruginosa* in a clinical setting are required to assess this combination's interactions.

Synergism of ciprofloxacin with other fluoroquinolones

As mentioned, the efflux pump is one of the main resistance mechanisms of bacteria against CIP. In this concept, Pankey et al. proposed that gatifloxacin, an 8-methoxyfluoroquinolone, could boost CIP's efficacy by inhibiting the efflux pump. Synergy testing was performed by E-test and time-kill assay for 31 clinically one kind, plasmid DNA distinct, *P. aeruginosa* segregates. Based on the E-test method, ciprofloxacin and gatifloxacin combination demonstrated synergy in six (19%) out of 31 *P. aeruginosa* isolates utilizing a summation fractional inhibitory concentration (FIC) of $\leq$0.5 for synergy. Also, the time-kill assay illustrated synergy for 13 (42%)/31 isolates*. Hence, it seems gatifloxacin inhibits the efflux pump and increases the efficacy of ciprofloxacin against *P. aeruginosa*; however, *in vitro* synergy by ciprofloxacin plus gatifloxacin against MDR *P. aeruginosa* should be evaluated in clinical setting.

Synergism of ciprofloxacin with cephalosporins

The combination use of cephalosporins with different FQ such as ciprofloxacin was considered by researchers. Mayer et al., reported that the combination of ciprofloxacin, ofloxacin, and pefloxacin with ceftazidime, examined by disc diffusion method, demonstrated synergy for only 3–5 isolates*. The three ciprofloxacin-β-lactam combinations, including ciprofloxacin + ceftazidime, ciprofloxacin + aztreonam, and ciprofloxacin + azlocillin, were evaluated against MDR isolates of *P. aeruginosa*. The frequency of synergy was subordinate to antibiotic susceptibilities. Based on the evidence, in case the organism was resistant to ciprofloxacin, synergy was found in more than 50% of the isolates, but if the organism was resistant to the β-lactam (excluding ceftazidime), synergy was commonly observed in <10% of the isolates*.

Synergism of ciprofloxacin with carbapenems

The combination of meropenem and ciprofloxacin seems more effective than either antibiotic alone in ICU infections due to *P. aeruginosa* strains. An earlier study examined 32 nosocomial-acquired *P. aeruginosa* strains between April 2001 and November 2001. Following the combination of ciprofloxacin with meropenem, an FIC index proposed synergy in two (6.2%) strains. The first strain was susceptible to ciprofloxacin but resistant to meropenem and imipenem; however, the second strain was both ciprofloxacin and carbapenems susceptible. Synergistic activity utilizing ciprofloxacin and imipenem happened in only one (3.1%) strain, which was susceptible to ciprofloxacin and imipenem*.

Time-kill synergy trials suggested that at 24 h, the sub-inhibitory meropenem and ciprofloxacin concentrations of 0.06–128 and 0.03–32 mg/L, respectively, indicated synergy against 34/51 *P. aeruginosa* strains, but that of 0.25–2 and 0.12–16 mg/L, respectively showed synergy against 18/52 *Acinetobacter baumannii* strains at the same period (164).

Rees et al. assessed bacterial killing and resistance suppression by combining meropenem with ciprofloxacin against *P. aeruginosa* in isolates collected from CF patients. Monotherapy with either meropenem or ciprofloxacin had a failure to suppress bacterial regrowth and the resistance of a hyper mutable clinical CF isolate at a high inoculum. However, the combination of 6 g of meropenem with 1.2 g of ciprofloxacin daily, both given periodically, achieved synergistic killing and resistance suppression over 8 days (165).

In another investigation, the authors separated two strongest extensive drug-resistant strains of *P. aeruginosa*, VIT PC 7 and VIT PC 9, from diabetic foot ulcer patients and tested their various resistance models utilizing whole genome sequencing. Susceptibility studies were applied using broth microdilution assay showing the impact of meropenem/ciprofloxacin susceptibility at higher concentrations, paving the way to design combinational drug examinations against these extensive drug resistance strains. The drug influence was significantly superior when the meropenem was utilized in combination with ciprofloxacin against VIT PC 7 and VIT PC 9, representing the increase of drug susceptibility by fourfold and eightfold (166).

In study performed by Pankuch et al. ciprofloxacin-meropenem combination was tested against 40 strains of *A. baumannii*. The micrograms per milliliter (MICs) of the antibiotics alone were as follows: ciprofloxacin 0.06–256 and meropenem 0.12–256. Ciprofloxacin plus meropenem, at 3 h, yielded synergy at sub-inhibitory concentrations (MICs) of ciprofloxacin (0.12 to 0.25) and meropenem (0.25) for two strains. At 24 h, the antibiotics indicated synergy against 18 strains at sub-inhibitory ciprofloxacin and meropenem concentrations of 0.12–16 and 0.25–2 μg/ml, respectively (164).

In other study by Lu et al. the *in vitro* antibacterial activity of meropenem combined with ciprofloxacin, was tested against clinically isolated XDR *A. baumannii*. The main actions of ciprofloxacin combined with meropenem were additive (56%) and indifference (44%) with synergistic and antagonistic effects (167). In the study of Sun et al. time-kill assay and checkerboard assay were conducted to study the combination effects *in vitro*. There was only one strain of *A. baumannii* for which ciprofloxacin plus meropenem indicated synergistic effect (168).

About *Enterobacteriaceae* spp. for example, in study performed by Ramadan et al. meropenem– ciprofloxacin combination showed indifferent effect ($n = 52$, 100%) on all carbapenem-resistant *K. pneumoniae* isolates, while meropenem–colistin combination indicated 25% synergism, and 59.6% indifference (169). Also in the study of Karki et al. the extensively drug resistant (XDR) isolates were tested for antimicrobial synergy and the results were interpreted as additive, synergistic, indifferent or antagonistic determining fractional inhibitory concentration (FIC) of the antibiotics. These isolates comprised *E. coli*, *K. pneumoniae*, *Acinetobacter baumannii*, and *P. aeruginosa*. All of the XDR isolates indicated "indifference" to the combination of meropenem- ciprofloxacin whereas few isolates indicated "antagonism" when tested with amikacin- ciprofloxacin and meropenem-colistin (170).

Synergism of ciprofloxacin with other antibiotics

A combination of colistin with either of tobramycin or ciprofloxacin has displayed synergism (45.45%; five out of 11 isolates) against MDR *K. pneumoniae* isolates (171). The isolates' MICs ranged from 0.25 to 32 $\mu g\ ml^{-1}$ for fosfomycin and from 1 to 1,024 $\mu g\ ml^{-1}$ for CIP. The combination of fosfomycin with ciprofloxacin reflected 6% synergy on biofilm formation by MDR urinary isolates of *E. coli*. The combination also diminished the MIC of each antibiotic (22).

Synergistic interplays (interplays indices 0.69–0.83; $P < 0.05$) were found between amphotericin B (0.07–0.31 mg/L) and either ciprofloxacin (0.19–7.65 mg/L) or levofloxacin (0.41–32.88 mg/L) against *Candida albicans* and *Aspergillus fumigatus*. Synergy (interplays indices 0.56–0.87; $P < 0.05$) was also discovered between voriconazole (0.09–0.14 mg/L) and ciprofloxacin (0.22–11.41 mg/L), as well as between caspofungin (8.94–22.07 mg/L) and levofloxacin (0.14–5.17 mg/L) against *A. fumigatus*. Ciprofloxacin could elevate the activity of antifungal agents against both *C. albicans* and *A. fumigatus* (172).

In vitro and *in vivo* examinations have highlighted that tigecycline in combination with ciprofloxacin is a powerful choice for treating invasive *Vibrio vulnificus* infection. An *in vitro* time-kill assay manifested synergism between tigecycline and ciprofloxacin. The survival rate was remarkably higher in mice treated with tigecycline plus ciprofloxacin than those treated with cefotaxime plus minocycline. Vancomycin-ciprofloxacin combination can be synergic against enterococci resistant to both vancomycin and ciprofloxacin. Still, it would be unlikely to have any excellence in treating enterococcal infections due to the high concentrations needed (173).

Synergism of ciprofloxacin with nanoparticles

Nanoparticles (NPs), particles with a size of 1–1,000 nm (commonly 5–350 nm in diameter), are made of any biocompatible substance. Different studies have reported acceptable antibacterial activity for NPs even against biofilm community of bacteria (174). To this end, the combined use of NPs with different antibiotics such as ciprofloxacin was considered by researchers for inhibition of bacterial growth and elimination of the biofilm community of these microorganisms.

In this concept, in the recently published study the authors synthesized embelin (Emb, isolated from *Embelia tsjeriam-cottam*)-chitosan-gold NPs (Emb-Chi-Au) were evaluated for their potential synergistic activity with ciprofloxacin by checker boarding assay and time-kill curve analysis. The NPs diminished the MIC of ciprofloxacin by 16- and 4-fold against MDR *P. aeruginosa* and *E. coli* strains, respectively. Furthermore, FIC records with ≤ 0.5 values affirmed the synergy between the ciprofloxacin and Emb-Chi-Au NPs, further confirmed at ½ MICs in both *P. aeruginosa* and *E. coli*, using time-kill curve analysis. In addition, Emb indicated the efflux pump-inhibitory potentials against both the organisms under consideration. Hence, the synergistic application of ciprofloxacin with Emb-Chi-Au NPs showed inhibitory impacts on two of the most MDR bacteria. To this end, the authors proposed that the inhibition of bacterial efflux pumps by NPs must have retained the concentrations of ciprofloxacin inside the cell, which acted against bacterial DNA topoisomerase/gyrase (175).

In addition to mentioned NPs, AgNPs are used in different studies to enhance ciprofloxacin efficacy. In one of these studies, the authors surveyed the synergistic bactericidal impact of AgNPs and ciprofloxacin on different bacteria such as *Pseudomonas solanocearum*, *Pseudomonas syringae*, *Xanthomonas malvacearum*, and *Xanthomonas campestris*. When 0.2 mM of AgNPs were combined with 1 μg of ciprofloxacin, the antiphytopathogenic activity was surprisingly expanded to 36, 40, 33, and 35 mm against all the mentioned bacteria, respectively. Similarly, MIC and minimum bactericidal concentration (MBC) values were diminished significantly, indicating the synergistic activity between AgNPs and ciprofloxacin (176).

In line with these results, Nikparast et al. reported that the combined antibacterial activity of ciprofloxacin with AgNPs

declined the MIC of antibiotics from 0.125 to 0.0625 μg/ml toward *P. aeruginosa*. Ciprofloxacin MIC against *P. syringae* reduced from 0.25 to 0.0625 μg/ml in combination with 6.25, 12.5, and 25 μg/ml of AgNPs (177).

In addition to AgNPs, zinc oxide (ZnO) was another metal-NPs that was used in combination with ciprofloxacin for enhancement of antibacterial activity. To this end, the authors synthesized ZnONPs, functionalizing them by Glu and conjugating them with thiosemicarbazid (TSC) to increase their efficacy against ciprofloxacin -resistant *S. aureus*. The results showed the synergistic activity of ciprofloxacin and synthesized NPs against ciprofloxacin -resistant *S. aureus*. Thus, the authors introduced ZnO@Glu–TSC NPs as a promising new antibacterial agent for therapeutic and preventive purposes (178).

The exact interaction of metal-NPs and ciprofloxacin has not been reported yet. However, it seems that these NPs, after attachment to the bacterial cell membrane, lead to the formation of gap on the bacterial cell walls, and damage to the cell membrane, thereby allowing the ciprofloxacin to enter the periplasm of the bacterial cells. Therefore, the combination of ciprofloxacin and metal NPs yield novel antimicrobial agents with synergistic properties that could be exploited for higher antibacterial activity. However, due to the high toxicity of these NPs for human cells, further investigation needs to be performed to evaluate the safety of these NPs for medical applications.

It's noteworthy to mention that, other studies that have used of nanoplatforms for enhancement of ciprofloxacin efficacy are presented in Table 4. Based on this table and mentioned studies, ciprofloxacin delivery can be modified by encapsulating with or incorporating different polymeric NPs such as poly lactic-co-glycolic acid (PLGA), chitosan, arginine, albumin, and other organic and inorganic nanostructure systems (179). Furthermore, studies have also shown that nano-platforms could enhance the efficiency of ciprofloxacin against bacterial cells, interfere with the biofilm community, enhance the penetration and protect the drug from deactivation or efflux (Figure 4).

Synergism of ciprofloxacin with natural products

Recent studies indicate that new antimicrobial agents are required to reduce the toxicity of conventional antimicrobial agents. Furthermore, combination therapy could improve the efficacy of different antimicrobials (199). In this regard, the combined ciprofloxacin and different natural products were considered to inhibit bacterial growth.

The recently published study used the checkerboard microdilution and evaluated *in vitro* interaction between *Thymbra spicata* L. extracts and certain antibiotics such as amikacin, cefotaxime, ampicillin, and ciprofloxacin against MDR *K. pneumonia* and *S. aureus*. The combination of amikacin, cefotaxime, and ampicillin plus plant extraction showed synergistic activity against S. *aureus*. In contrast, the joint activity of plant extract with ciprofloxacin indicated indifferent and additive activity. Furthermore, ciprofloxacin showed an indifference and additive effect with sensitive and resistant *K. pneumoniae* strains when combined with all *T. spicata* extracts (200).

Based on the checkerboard synergy technique, nbutanolic *Cyclamen coum* extract in combination with ciprofloxacin represented a synergistic effect against *P. aeruginosa* biofilms (ΣFBIC = 0.496) (201). The extricates of four customarily utilized therapeutic plants, i.e. *Plumbago zeylanica* (root), *Hemidesmus indicus* (stem), *Acorus calamus* (rhizome), and *Holarrhena antidysenterica* (bark), were examined against the clinical isolates of MRSA and methicillin-sensitive *S. aureus*, *P. zeylanica* and *H. antidysenterica* demonstrated synergism with ciprofloxacin (202).

Additionally, the MIC findings of another investigation uncovered that combinatorial impacts of Sami-Hyanglyun-Hwan ethanol extract (SHEE) with ciprofloxacin had 2–32-fold reduction in concentration as those needed by SHHE alone. The antibacterial activity of SHHE obviously declined the MICs of ciprofloxacin against *S. aureus* strains. The checkerboard method suggested that the combinations of SHHE with ciprofloxacin had a partial methicillin-resistant synergistic or synergistic impact on MRSA. The time-kill curves also proved that *S. aureus* in combination with SHHE and ciprofloxacin treatment, lessened the bacterial counts significantly after 24 h (203). Chrysoeriol had a notable synergistic impact when combined with ciprofloxacin and oxacillin against epidemic methicillin-resistant *S. aureus* 15 (EMRSA-15) and EMRSA-16, respectively, both of which are the UK epidemic MRSA strains (204). When biochanin A (BCA) was combined with ciprofloxacin, the FIC index data exhibited that there was synergy in all 12 of the *S. aureus* strains examined. The outcomes of time-kill tests and agar diffusion tests affirmed synergy between BCA and ciprofloxacin against *S. aureus* strains. These results proposed that BCA can be combined with FQs to produce a potent antimicrobial agent (205).

On the other hand, the results of another study showed that the combination of Propolis, a mixture of a complex chemical composition containing essential oils, balms, pollen, minerals, vitamins, and proteins, with ciprofloxacin has shown an antagonistic effect against MRSA. The ciprofloxacin action is diminished when combined with Propolis. In five of the seven strains studied, further growth of MRSA was found in combinations in concentrations of each substance separately applied. Hence, the combination of both substances is noxious (206).

Thus, combining ciprofloxacin with natural products could lead to several advantages such as boosted potency, a reduced dose of drugs needed and minimized toxicity, which ultimately

TABLE 4 The studies have used nano-platform for the enhancement of ciprofloxacin against different bacteria.

References	Nanoplatforms for delivery of ciprofloxacin	Bacteria	Outcomes
(180)	Ciprofloxacin-AgNPs	*A. baumannii* *S. marcescens* *S. aureus*	Compared to ciprofloxacin alone, this compound showed better antioxidant, anti-biofilm, and antibacterial function against the pathogenic bacteria tested
(181)	Chitosan/dysprosium oxide	NA	This nanocomposite has good potential for a controlled drug delivery system
(182)	Synthesized red blood cell membrane-coated PLGA	*K. pneumoniae*	This NP showed good antibacterial and anti-infection ability
(183)	Gelatin-sodium carboxymethyl cellulose composite nanogels	*S. aureus*	This compound showed antibacterial activity with sustained-release performances
(184)	Nano-fluid containing carbon nano-tubes	*Drug-resistant K. pneumoniae*	Simultaneous usage of nano-fluid and antibiotics could enhance antibiotic effectiveness at lower doses
(185)	Hemicelluloses from *Lallemantia royleana*, chitosan/chitin and glutaraldehyde	*S. aureus* *E. coli*	This compound showed comparable activity against *E. coli* to that of ciprofloxacin and relatively lower activity in the case of *S. aureus*
(186)	Graphene-silk fibroin macromolecular hydrogel dressings	*S. aureus* *P. aeruginosa*	This compound improved antibacterial activity against both bacteria and burn wound infection
(187)	Clay/alginate/imidazolium-based ionic liquid	*E. coli* *P. aeruginosa*	Ciprofloxacin-loaded nanocomposites showed significantly higher antibacterial activity in comparison with free ciprofloxacin
(188)	Hyaluronic acid functionalized self-nano-emulsifying drug delivery system	*Salmonella typhi*	The drug-delivery system with ciprofloxacin showed an improved ability to permeate goat intestinal mucus, antibiofilm activity, and oral pharmacokinetics compared to free ciprofloxacin
(189)	Ciprofloxacin-azithromycin NPs on chitosan nanocarriers	*P. aeruginosa*	This compound significantly inhibited the biofilm community of bacteria in comparison to the free ciprofloxacin
(190)	Chitosan microspheres/nano hydroxyapatite-titanium	*S. aureus*	Showed antibacterial activity
(191)	Citric acid cross-linked carboxymethyl guar gum nanocomposite films	NA	Enhanced the wound healing
(192)	Sodium alginate cross-linked with nano-hydroxyapatite	*P. aeruginosa* *S. aureus* *E. coli*	Showed antibacterial, especially against *S. aureus*
(193)	Poly(DL-lactide-co-glycolide) NPs	*P. aeruginosa* *S. aureus*	The NPs were safer and more effective against bacteria in comparison to free drugs
(194)	poly(vinyl alcohol) /citric acid/Ag NPs	*S. aureus* *E. coli*	Showed an effective antibacterial activity.
(195)	Fe_3O_4@ polyacrylic acid @ZIF-8	*S. aureus* *E. coli*	This compound decreased the growth of bacteria
(196)	Zn containing mesoporous silica nanospheres into polycaprolactone electrospun fibers	*E. coli*	Showed antibacterial and wound healing capacity
(197)	Cerium-doped nano-bioactive glasses	*P. aeruginosa* *S. aureus* *E. coli* *Bacillus subtilis*	Showed antibacterial activity against all studied bacteria
(198)	Nano gold embedded cellulose grafted polyacrylamide nanocomposite hydrogel	*E. coli* *Shigella flexneri* *Bacillus cereus* *Listeria Inuaba*	This nanocomposite with improved rheological and thermal characteristics is suitable and proposed as a good carrier for *in vitro* release of ciprofloxacin drugs

NPs, nanoparticles; NA, not applicable; NR, not reported.

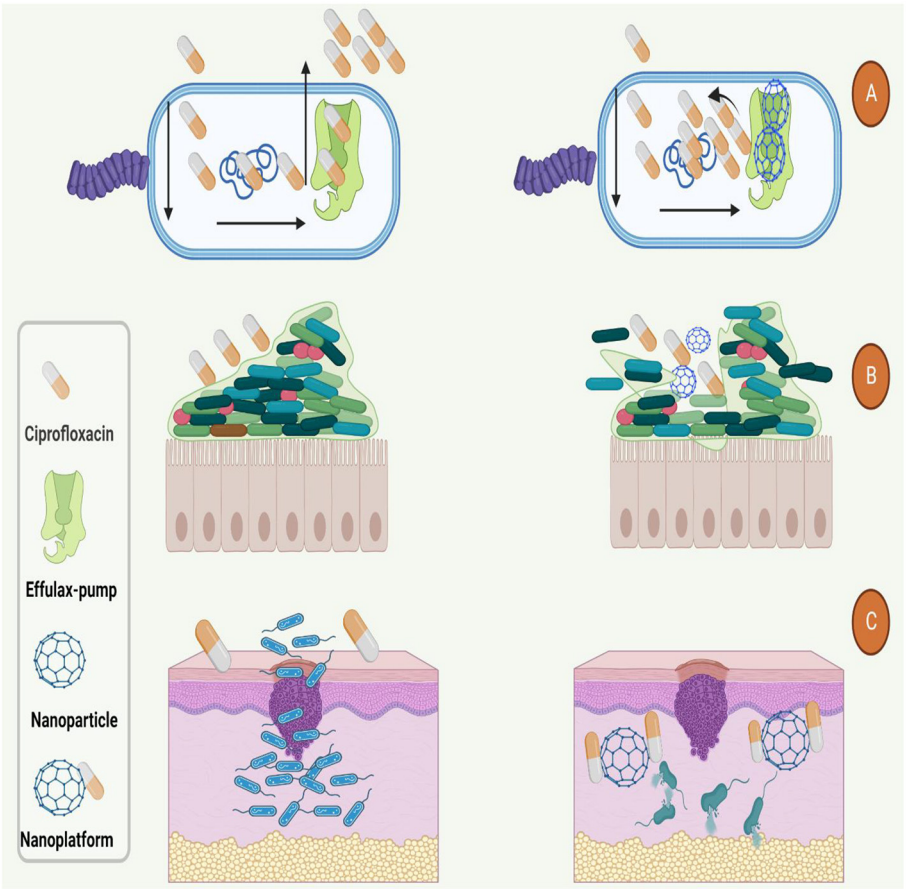


FIGURE 4
Used nano-platforms for enhancement of ciprofloxacin efficacy against bacteria. **(A)** Nanoparticles could boost the antibacterial function of ciprofloxacin by inhibition of efflux-pumps. **(B)** Nanoparticles increase the antibacterial activity and penetration of ciprofloxacin to the **(B)** dipper layers of biofilm and **(C)** body organs such as the skin.

helps inhibit different bacteria even MDR isolated. Although, the exact mechanism by which natural products synergizes with ciprofloxacin was not investigated in the studies mentioned above. Therefore, additional molecular and *in vivo* studies are needed to confirm the practical utility of these combinations. Finally, in addition to natural products, the combined use of ciprofloxacin with various natural compounds, which have antimicrobial properties, such as curcumin, eugenol, cinnamomum, and carvacrol, should be considered.

Synergism of ciprofloxacin with photodynamic/laser therapy

Photodynamic therapy (PDT) has been identified as an effective treatment for the inhibition of bacterial infections such as *E. faecalis* infection in root canal dentine (207). In this method, a specific wavelength excites a photosensitizer, photoactive dye, and leads to the generation of singlet oxygen or other reactive oxygen species (ROS) that can eliminate the target bacteria (208). Methylene blue (MB), due to various characteristics such as low molecular weight and toxicity in mammalian cells, and hydrophilicity, are reported as a potential photosensitizer for PDT (209). In recent years, different methods have been used to enhancement of PDT efficacy for the inhibition of bacterial infections. The use of antibiotics in combination with PDT is one of these methods. In this regard, researchers used ciprofloxacin to boost the performance of the PDT.

To this end, the findings of the recently published study showed that *S. aureus,* even with the lowest ciprofloxacin and MB concentrations (0.0625 and 6.25 μg/mL, respectively), bacterial killing was remarkably developed when compared to MB–PDT alone for the exact light dose. The best findings were achieved after the combination treatment of PDT with, followed by ciprofloxacin on biofilms, which enhanced bacterial diminishment on biofilms, resulting in a 5.4 log diminishment for *S. aureus* biofilm and approximately seven logs for *E.*

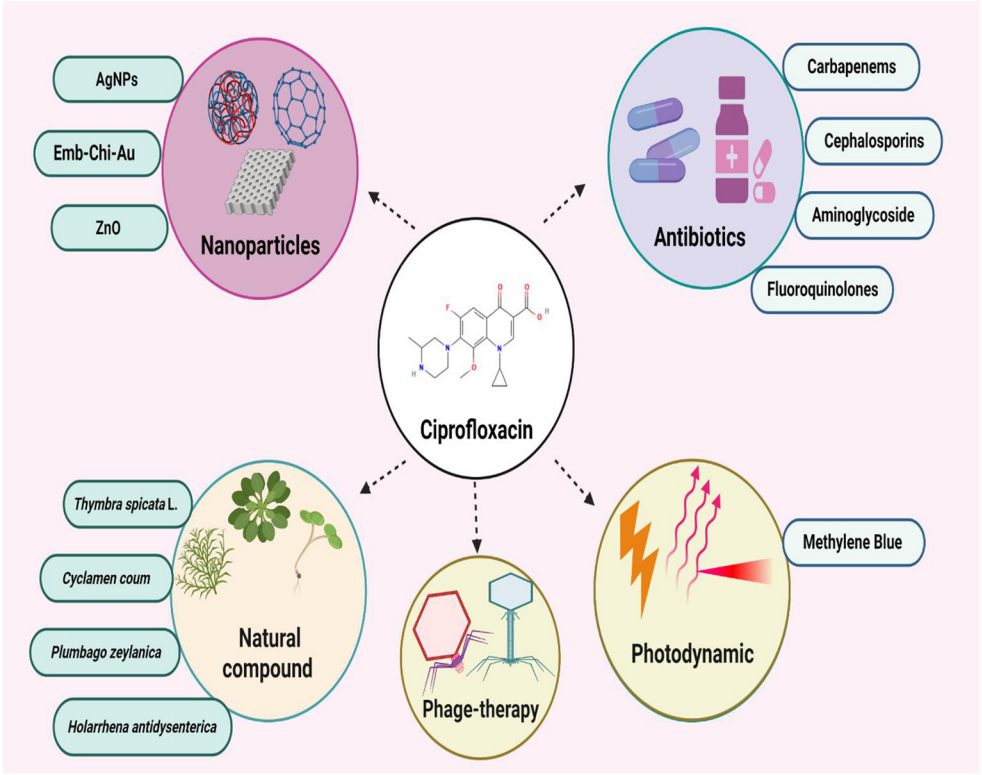


FIGURE 5
The combination uses ciprofloxacin and other antibacterial agents.

coli biofilm (210). In another investigation also, the authors reported that essential oil obtained from *Eugenia jambolana* interferes with the action of antibiotics against bacteria exposed to LED lights. This trial showed that irradiation of *E. coli* and *S. aureus* with blue or red light in the presence of ciprofloxacin is more beneficial than antibiotic monotherapy (211). Therefore, PDT can destroy the bacterial community using various possible mechanisms such as interference with cellular hemostasis and membrane permeability, modulation of DNA and RNA synthesis, and alkalization of the cytoplasm and cell membrane depolarization. In this regard, the combine use of PDT and ciprofloxacin can be considered for treatment of bacterial infections especially infection that caused by MDR bacteria; however, the data about this kind of treatment is very limited and more confirmatory studies are needed.

Synergism of ciprofloxacin with bacteriophages

Bacteriophages (phages), viruses that infected bacteria, were first discovered in the middle of the 20th century, and due to their great function in the inhibition of MDR bacteria, was considered by scientist as non-antibiotic approaches for the treatment of bacterial infections. Eukaryotic cells have no receptors for phages; therefore, they can be used to treat bacterial infections (212). A phage cocktail containing two or more bacteriophage mixtures with different host ranges in a single suspension could lead to a better antibacterial effect than single phage therapy (213, 214). Phages could penetrate the dipper layer of biofilm and damage its structure by producing natural enzymes. Additionally, endolysins are produced at the end of the lytic cycle of the phages. This enzyme could destroy bacterial cell walls by the cleavage of peptidoglycan (215, 216). To this end, combination therapy of antibiotics and phage not only causes a reduction in the number of bacteria but also can be related to the management of phage-resistant bacteria levels (217). Therefore, this section will discuss the combination therapy of ciprofloxacin and phage for treating bacterial infections (Figure 5).

Recently published studies reported additive or synergistic effects of ciprofloxacin and phage combination (134, 218–223). Gurney et al. reported that phages could interact with different structures of *P. aeruginosa,* such as lipopolysaccharide structure (LPS) and the efflux pumps. Therefore, phages could increase the permeability of bacteria and the drug dosage by inhibiting

TABLE 5 Studies have used various approaches to enhance ciprofloxacin activity against bacterial infection in animal models and *in vivo*.

References	Antibacterial agents	Animal models	Bacteria	Outcome
(231)	Recombinant glycoside hydrolases	Lung infection	*P. aeruginosa*	The Co-T* leads to a greater reduction in pulmonary bacterial burden than with either agent alone
(232)	PDT with cationic imidazolyl photosensitizers	Wound infection	*E. coli*	This synergic combination decreased the ciprofloxacin and photosensitizer needed for full bacteria inactivation
(233)	Toll-like receptor 2 agonist	*B. anthracis* infected mice	*B. anthracis*	The Co-T showed augmented activity in protecting mice from infection
(234)	Non-hydroxamate LpxC inhibitor	Murine model of pneumonia	*K. pneumoniae*	The Co-T decreased the production of IL-6 and LPS release induced by ciprofloxacin in the lung
(235)	Macrophage-membrane NPs	Mouse peritoneal infection model	*S. aureus*	NPs killed staphylococci more effectively than ANPs without membrane encapsulation
(236)	Neutrophil-factor S100A8/A9	Biofilm-infected chronic wounds	*P. aeruginosa*	Ciprofloxacin monotherapy developed resistance (after 14 days), while combination therapy changed the resistance pattern
(237)	Ciprofloxacin/rolipram nanostructured lipid carriers	Bacteremia with organ injury	MRSA	This compound remarkably reduced elastase distribution and MRSA burden in the organs of MRSA-infected animals
(238)	Thymine	Galleria mellonella infection model	*E. coli*	Thymine significantly enhanced ciprofloxacin activity
(221)	Phage	Neutropenic mouse model of acute lung infection	*P. aeruginosa*	The Co-T remarkably decreased the bacterial load in mouse lungs. In contrast, no significant reduction in the load of bacteria was detected when the animals were treated only with phage or ciprofloxacin
(239)	Truncated alpha-defensins analog 2Abz23S29	Murine model of urinary tract infection	UPEC	The macrophage inflammatory protein/2 and IL-6 in infected mice treated with combination therapy were remarkably higher than in the untreated mice
(240)	Antibiotic-loaded adipose-derived stem cells	Rat implant-associated infection model	*S. aureus*	Rats treated with combination therapy had the lowest abscess formation, modified osteomyelitis scores, and bacterial burden on the implant
(241)	PLGA microsphere-based composite hydrogel-ginsenoside Rh2	Mouse model of MRSA skin infections	MRSA	Great potential for the treatment of wound infection
(242)	2-(2-aminophenyl) indole (efflux pump inhibitor)	Murine thigh infection model	*S. aureus*	The Co-T indicated significant efficacy against bacterial infection
(173)	Antibiotics	Invasive infection	*Vibrio vulnificus*	The survival rate was significantly higher in mice treated with tigecycline plus ciprofloxacin than in mice treated with cefotaxime plus minocycline
(243)	Glycyrrhizin	Ocular infection	*P. aeruginosa*	The Co-T vs. ciprofloxacin remarkably decreased plate count, clinical scores, and myeloperoxidase
(244)	3-hydroxypyridin-4-one chelator	Pneumonia	*Acinetobacter baumannii*	Treatment with ciprofloxacin alone was insufficient for removing infection caused by ciprofloxacin-resistant bacteria; however, the combination therapy significantly improved treatment efficacy
(245)	Immunomodulatory S100A8/A9	Murine chronic wound model	*P. aeruginosa*	Augmented the effect of ciprofloxacin

PDT, photodynamic therapy; Co-T, combination therapy; LPS, lipopolysaccharide; NPs, nanoparticles; MRSA, methicillin-resistant Staphylococcus aureus; UPEC, uropathogenic E. coli.
*Combination therapy of antibacterial agent with ciprofloxacin.

efflux pumps (134). Another study also indicated that combining phage cocktail and ciprofloxacin could increase the number of MDR *P. aeruginosa* strains' susceptibility to this antibiotic. This combination therapy also resulted in the re-sensitization of *P. aeruginosa* to ciprofloxacin. Noteworthy, the animal wound model result showed that phage-only treated mouse wounds had mutations for phage receptors; thus, these animals were resistant to infection with phage. However, these mutations were not detected in the combination treatment bacteria, suggesting that the treatment with phages and antibiotics reduced the incidence of the bacteria becoming resistant to the phage treatment (218). In another investigation, the authors reported that intratracheally treating mice (with acute lung infection) with phage- ciprofloxacin combination powder remarkably decreased the bacterial load in the lungs. In contrast, single treatments failed to reduce the bacterial count (221).

Therefore, the combination use of phage–antibiotic is a promising approach for in inhibition of MDR bacteria, especially *P. aeruginosa*. The phage- ciprofloxacin synergistic effect in killing bacterial cells could be due to a selective pressure under which the bacteria mutate in one trait to improve fitness while suffering a decrease in another trait. A recently published study reported an evolutionary trade-off effect when phage treatment imposed a selective pressure on MDR bacteria. When bacteria lose their receptor for phage binding, they resist to infection by phages. However, in this condition, bacteria regained sensitivity to a different antibiotic, such as ciprofloxacin. Another possible reason might be morphologic changes of bacterial cells when exposed to sub-inhibitory concentrations of antibiotics. In this circumstance, antibiotic exposure led to the elongation of bacterial cells but did not divide, which could improve phage assembly and maturation (221, 224, 225).

Additionally, as mentioned in previous parts of the manuscript, the biofilm community of bacteria is one of the most important challenges in treating infection. Given that, the combination uses of ciprofloxacin and phages have been considered by scientists for the elimination of bacterial biofilm. Tkhilaishvili et al. reported that a higher concentration of ciprofloxacin is required to suppress the growth of dual-species biofilms compared to monospecies biofilms. On the other hand, combining phages with ciprofloxacin significantly enhanced the anti-biofilm activity of both antimicrobials with complete eradication of *S. aureus*/*P. aeruginosa* biofilms (226). In line with these findings, a recently published study also reported that antibiotics such as ciprofloxacin and phages alone had a modest effect in killing bacteria in biofilm community.

Nonetheless, when these compounds were used at the same time, especially when ciprofloxacin was added sequentially after 6 h of phage treatment, a significant enhancement in the killing activity was detected (227). It seems phages *via* depolymerases could degrade the biofilm matrix, consequently enhancing antibiotic penetration into the deeper layers of the biofilm (227–229). However, depolymerases was not detected in some phages; therefore, the mentioned phenomenon might not have been responsible for the synergistic action of the phages and antibiotics combined therapy. In these cases, it's possible that phages using of biofilm void spaces could access the dipper layers of the biofilm. Afterward, phages replicate in the biofilm's deeper layer and interrupt the biofilm's extracellular matrix. The addition of antibiotics following this interruption causes an improved bacterial killing due to the deeper penetration of phages and antibiotics (227, 230).

Taken together, combining ciprofloxacin with phages can be synergistic in destroying the bacteria in the biofilm community; hence, this combination therapy is a promising candidate for treating infections are caused by MDR bacteria. However, some important challenges, such as the time of antibiotic application, the concentration of antibiotics, and the exact interaction of phages with eukaryotic cells, should be evaluated in further studies.

Finally, its noteworthy that recently published studies that have used various antibacterial agents to enhance ciprofloxacin efficacy against different bacterial infections in animal models and *in vivo* studies are presented in Table 5.

Conclusion

Ciprofloxacin's potential for the treatment of a large spectrum of bacterial infections led to the overuse of this drug in clinical practice and developed alarming levels of ciprofloxacin resistance as a consequence of heavy use. To preserve this beneficial agent, prescribers must ensure that ciprofloxacin is a proper choice and administer enough doses to limit the risk of selecting resistant mutant bacterial subpopulations. The increasing incidence of ciprofloxacin-resistant pathogens jeopardizes the continued empiric use of ciprofloxacin and raises the urgent need to develop novel ciprofloxacin derivatives potent against both drug-susceptible and drug-resistant pathogens and discover useful synergism between ciprofloxacin and other antibacterial agents. As mentioned earlier in this study, recent studies have reported a wide range of synergism between ciprofloxacin and other antibacterial agents. Therefore, the combination use of ciprofloxacin and other antibiotics and antibacterial agent should be considered in future studies because combination therapy could increase antibacterial performance of ciprofloxacin especially against MDR strains.

Author contributions

SK and MH conceived and designed the study. AS, MAr, MK, MAb, MG, MH, and SK contributed in comprehensive research. AS and MH participated in editing the manuscript. All authors have read and approved the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Degenerate Beta autoregressive model for proportion time-series with zeros or ones: An application to antimicrobial resistance rate using R shiny app

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Background: Antimicrobial resistance has emerged as one of the foremost public health troubles of the 21st century. This has ended in a public health disaster of the global situation, which threatens the exercise of present-day remedy. There is an urgent requirement for a cost-effective strategy to reduce antimicrobial resistance. Infectious disease control researchers most often analyze and predict antimicrobial resistance rate data that includes zeros or ones. Commonly used time-series analysis such as autoregressive moving average model is inappropriate for such data and may arrive at biased results.

Objective: This study aims to propose a time-series model for continuous rates or proportions when the interval of series includes zeros or ones and compares the model with existing models.

Data: The *Escherichia coli*, isolated from blood cultures showing variable susceptibility results to different antimicrobial agents, has been obtained from a clinical microbiology laboratory of a tertiary care hospital, Udupi district, Karnataka, during the years between 2011 and 2019.

Methodology: We proposed a Degenerate Beta Autoregressive model which is a mixture of continuous and discrete distributions with probability mass at zero or one. The proposed model includes autoregressive terms along with explanatory variables. The estimation is done using maximum likelihood with a non-linear optimization algorithm. An R shiny app has been provided for the same.

Results: The proposed Degenerate Beta Autoregressive model performed well compared to the existing autoregressive moving average models. The forecasted antimicrobial resistance rate has been obtained for the next 6 months.

Conclusion: The findings of this article could be beneficial to the infectious disease researchers to use an appropriate time-series model to forecast the resistance rate for the future and to have better or advance public health policies to control the rise in resistance rate.

KEYWORDS

Beta distribution, time-series model, mixture distribution, rates, proportions, inflated distribution, AMR, resistance

1. Introduction

Antimicrobial resistance (AMR) is a serious problem in many developing countries. The World Health Organization (WHO) has categorized AMR as a serious health problem affecting the patients of various countries. In 2015, the WHO surveyed in its six regions called country situation analysis to examine the current practices and to determine the gaps which increase antimicrobial resistance. Eleven and eight countries from Asian and African continents, respectively, from low- to middle-income participated in the study. The analysis showed that AMR is a major issue in both the continents, and in the South-East Asia, nosocomial infections are of particular concern. The main cause of resistance is the inappropriate use of antimicrobial medicines and poor healthcare facilities (1).

In the past, to study antibiotic usage and resistance time-series, models such as Box-Jenkins ARIMA (autoregressive-integrated moving average) (2) and transfer function models have been used. Some studies used time-series to forecast trend and seasonality of the data with the exponential smoothing technique.

However, there is a limitation of these models in terms of accuracy of prediction or violation of assumptions in the applicability. Hence, this study proposes to develop and test stochastic models to predict antibiotic resistance rate, which helps us in understanding the pattern of resistance to plan strategies for the rational use of antibiotics.

Oftentimes, the data like antimicrobial resistance include data in the interval [0, 1) or (0, 1], when the bacteria is highly susceptible or resistant to the antibiotic. To model data with rates/proportions regression, models are proposed by Ferrari and Cribari-Neto (3), Mitnik and Baek (4), Pumi et al. (5), and Artur and Bazán (6) and the time-series models are proposed by Rocha and Cribari-Neto (7) and Bayer et al. (8). But these models are not appropriate for the proportion data with zeros or ones. To model such kind of data, we look for a mixture of distributions. Ospina and Ferrari (9) and Cribari-Neto and Santos (10) introduced inflated Beta distributions and inflated Kumaraswamy distributions which is a mixture of discrete and continuous distributions. Ospina and Ferrari (11) and Bayer et al. (12) introduced the inflated Beta regression model and inflated Kumaraswamy regression model. Currently, there is scope to use a time-series model for proportion data in the interval [0, 1) or (0, 1].

This paper proposes to model the time-series data in the interval [0, 1) or (0, 1] using a mixture of Degenerate and Beta distributions through a frequentist approach. This study is an extension of βARMA model proposed by Rocha and Cribari-Neto (7), where instead of Beta distribution, inflated Beta distribution is incorporated. The proposed model is compared with the ARIMA model.

The developed model is illustrated with an application based on antimicrobial resistance (AMR) data. Rates of *Escherichia coli* (*E. coli*) isolated from blood cultures showing variable susceptibility results to different antimicrobial agents are considered for the modeling in this study. *E. coli* is a gram-negative bacteria, most regularly isolated in patients with blood stream infection (BSI), and in severe instances, it may cause loss of life. The rates of BSI have accelerated steadily in recent years (13). However, knowledge of future resistance rate using forecasting may help in recommending new interventions or policy recommendations in hospital settings.

2. Materials and methods

2.1. Data description

The variable susceptibility of *Escherichia coli* (*E. coli*), isolated from blood cultures showing variable susceptibility results to different antimicrobial agents, has been obtained from a clinical microbiology laboratory of a tertiary care hospital, Udupi district, Karnataka, during the years between 2011 and 2019. Institutional ethical clearance was obtained from Kasturba Medical College and Kasturba Hospital Institutional Ethics Committee (IEC no. 832/2019). The laboratory generally receives more than 10,000 blood culture tests annually from patients who have been suspected of bacteremia/sepsis. The blood cultures yielded positive results in 20–30% of cases and *E. coli* is the most common among several other bacteria causing bacteremia in our patient population. We retrieved the data of antimicrobial susceptibility test (AST) results of this bacteria from the electronic records of the laboratory. Antimicrobial susceptibility tests were performed using the Kirby Bauer disc diffusion method until 2014 and by the Vitek-2 automated method after 2015, both of which are accepted and standardized test methods according to the Clinical Laboratory Standards Institute (Ref:CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute; 2012). In the former method, the bacteria was considered resistant to the antimicrobial agent amoxicillin-clavulanic acid, based on the ESBL phenotypic test, although the individual agent appeared susceptible. However, in the latter method, results were reported unchanged as detected in the automated system. Laboratory had tested *E. coli* against different antimicrobial agents and the results were interpreted as either susceptible or resistant. The data include information on the monthly number of *E. coli* isolates obtained and the total number of isolates resistant to the different antibiotics which includes a total of 108 time points. The resistance proportion is calculated as the number of *E. coli* isolates that were resistant to a particular antimicrobial agent/total number of *E. coli* tested during that time interval. This study considered the data on the antibiotic amoxicillin-clavulanic acid (AMC) and Cefoperazone-sulbactam (CSL) since it met the requirements

of the model (i.e., time dependency and in the interval [0,1) or (0,1]).

2.2. Degenerate Beta autoregressive (DeβAR) model

2.2.1. Beta distribution

The parameterized probability density function of random variable X of Beta distribution is

$$f(x;\mu,\zeta)=\frac{\Gamma(\zeta)}{\Gamma(\mu\zeta)\Gamma((1-\mu)\zeta)}x^{\mu\zeta-1}(1-x)^{(1-\mu)\zeta-1},0<x<1 \quad (1)$$

Here, $0<\mu<1$ and $\zeta>0$. The mean and variance of the random variable X is, $E(X=x)=\mu$ and $V(X=x)=\frac{V(\mu)}{1+\zeta}$; where $V(\mu)=\mu(1-\mu)$. Hence, here μ and ζ act as distribution mean and precision parameters. Here, when value of μ is fixed, as the value of ζ increases, the variance of x decreases.

2.2.2. Degenerate distribution

A Degenerate distribution is a one-point distribution where a random variable X has a single possible value.

The probability mass function of random variable X can be written as follows:

$$P(X=x)=\begin{cases}1 \text{ if } x=c;\\ 0 \text{ elsewhere}\end{cases} \quad (2)$$

That is, a random variable, X, is degenerate if, for some constant, c, P(X = c) = 1.

This distribution has a single parameter, c, and it ranges from - ∞ to ∞.

2.2.3. Inflated Beta distribution

Ospina and Ferrari (9) introduced inflated Beta distributions which is a mixture of Degenerate and Beta distributions. The probability density function is

$$bi_c(x;\omega,\mu,\zeta)=\begin{cases}\omega & \text{if } x=c;\\ (1-\omega)f(x;\mu,\zeta) & \text{if } x\in(0,1)\end{cases} \quad (3)$$

where, $0<\omega<1$ is the mixture parameter, $f(x;\mu,\zeta)$ is the p.d.f of Beta distribution and c=0 or 1 known value which follows Degenerate distribution. The cumulative distribution function (c.d.f) of mixture distribution is given by

$$BI_c(x;\omega,\mu,\zeta)=\omega\mathcal{I}_{[c,1]}(x)+(1-\omega)F(x;\mu,\zeta)$$

Where, $\mathcal{I}_A(x)$ is an indicator function that equals 1 if $x\in A$ and 0 if $x\notin A$. Here, $F(.;\mu,\zeta)$ is the c.d.f. of the beta distribution.

The r^{th} moment and variance of inflated beta distribution is

$$E(x^r)=\omega c+(1-\omega)\mu_r$$

$$Var(x)=(1-\omega)\frac{V(\mu)}{\zeta+1}+\omega(1-\omega)(c-\mu)^2$$

where, $\mu_r=(\mu\zeta)_r/\zeta_r$.

2.2.4. Beta autoregressive moving average model

Rocha and Cribari-Neto (7) introduced βARMA model to fit continuous time-series data in the interval (0, 1), which follows Beta distribution.

The proposed βARMA(p, q) model is

$$g(\mu_t)=\beta_0+s_t'\beta+\Sigma_{i=1}^{p}\phi_i\{g(x_{t-i})-s_{t-i}'\beta\}+\Sigma_{j=1}^{q}\alpha_j r_{t-j} \quad (4)$$

where, g(.) is the link function, β_0 is the intercept term, s_t's are the regressor variables, and $\boldsymbol{\beta}=(\beta_1,\beta_2,...,\beta_k)'$ are set of parameters of regressors. The ϕ's and the α's are the autoregressive (AR) and moving average (MA) parameters, p and q are the AR and MA orders, and r_t is an error term, respectively.

In case of a real-life scenario when time-series data includes zeros or ones, it is challenging to use this model by assuming Beta distribution. To model such kind of continuous time-series data, we replaced Beta distribution with inflated Beta distribution proposed by Ospina and Ferrari (9). The density function of inflated Beta distribution with time can be written as follows:

Let X_t t=1,2...n be the response variable of proportion data which includes zeros or ones. We assume that the proportion series is conditionally distributed as InBE (μ_t, ζ, ω) (where, InBE stands for "Inflated Beta") with probability density function defined as:

$$f_{X_t}(x_t|\mathcal{F}_{t-1})=\omega\mathcal{I}_{(x_t=c)}+(1-\omega)\frac{\Gamma(\zeta)}{\Gamma(\mu_t\zeta)\Gamma((1-\mu_t)\zeta)}x_t^{\mu_t\zeta-1}(1-x_t)^{(1-\mu_t)\zeta-1} \quad (5)$$

or equivalently

$$f_{X_t}(x_t|\mathcal{F}_{t-1})=\begin{cases}\omega & if x=c;\\ (1-\omega)\frac{\Gamma(\zeta)}{\Gamma(\mu_t\zeta)\Gamma((1-\mu_t)\zeta)}x_t^{\mu_t\zeta-1}(1-x_t)^{(1-\mu_t)\zeta-1} & if x\in(0,1)\end{cases}$$

which is a mixture of Beta and Degenerate distributions. Here, $\mathcal{F}_{t-1}$ is the previous information set of response series. When the mixture parameter $\omega=0$, inflated Beta distribution reduces

to Beta distribution. Based on Equations (3) and (4), the mean and variance of distribution can be written as follows:
For [0,1),

$$E(X_t|\mathcal{F}_{t-1}) = (1-\omega)\mu_t$$

$$V(X_t|\mathcal{F}_{t-1}) = (1-\omega)\frac{\mu_t(1-\mu_t)}{\zeta+1} + \{\omega(1-\omega)\mu_t^2\}$$

For (0,1],

$$E(X_t|\mathcal{F}_{t-1}) = \omega + (1-\omega)\mu_t$$

$$V(X_t|\mathcal{F}_{t-1}) = (1-\omega)\frac{\mu_t(1-\mu_t)}{\zeta+1} + \{\omega(1-\omega)(1-\mu_t)^2\}$$

2.2.5. Proposed model

The proposed Degenerate Beta Autoregressive (DeβAR) model for the parameters of mixture distribution is

$$\eta_t = g(\mu_t) = s_t'\beta + \Sigma_{i=1}^{p}\phi_i\{g(x_{t-i}) - s_{t-i}'\beta\} \qquad (6)$$

where, η_t is the linear or non-linear predictor of the model, where g(.) is the link function (we used logit), s_t's are the regressor variables, and $\boldsymbol{\beta} = (\beta_1, \beta_2, ..., \beta_k)'$ are the unkonwn parameters of regressor variables. $\boldsymbol{\phi_i} = (\phi_1, \phi_2, ..., \phi_p)'$ are the autoregressive parameters with order p. Let $\boldsymbol{\theta} = (\beta', \zeta, \omega, \phi_i')'$ be vector of unknown parameters with length k+p+2.

2.2.6. Parameter estimation

The parameters of the model are estimated by maximizing log-likelihood function.

Here, let X_t, t=1,2,...,n be a random variable and $\mathcal{F}_{t-1}$ be the set of past information. Then, the likelihood function of the parameters θ conditioning on the past p observations can be written as follows:

$$L(\boldsymbol{\theta}; x) = \prod_{t=p+1}^{n} f_{X_t}(x_t|\mathcal{F}_{t-1})$$

The likelihood function for the parameters of Degenerate Beta AR model is given by

$$L(\boldsymbol{\theta}; x) = \prod_{t=p+1}^{n}\{\omega\mathcal{I}_{x_t=c} + \mathcal{I}_{x_t\epsilon(0,1)}(1-\omega) \frac{\Gamma(\zeta)}{\Gamma(\mu_t\zeta)\Gamma((1-\mu_t)\zeta)}x_t^{\mu_t\zeta-1}(1-x_t)^{(1-\mu_t)\zeta-1}\}$$

Then, the log-likelihood of model is

$$log(L(\boldsymbol{\theta}; x)) = l(\theta) = \sum_{t=p+1}^{n}\mathcal{I}_{x_t=c}log(\omega) + \sum_{t=p+1}^{n}\mathcal{I}_{x_t\epsilon(0,1)}log\{(1-\omega) \frac{\Gamma(\zeta)}{\Gamma(\mu_t\zeta)\Gamma((1-\mu_t)\zeta)}x_t^{\mu_t\zeta-1}(1-x_t)^{(1-\mu_t)\zeta-1}\}$$

Here, take $\mathcal{I}_{x_t=c} = x_{ct}$, then $l(\boldsymbol{\theta})$ equals to

$$\sum_{t=p+1}^{n}x_{ct}log(\omega) + \sum_{t=p+1}^{n}(1-x_{ct})log\{(1-\omega) \frac{\Gamma(\zeta)}{\Gamma(\mu_t\zeta)\Gamma((1-\mu_t)\zeta)}x_t^{\mu_t\zeta-1}(1-x_t)^{(1-\mu_t)\zeta-1}\}$$

Then, the score function is given by

$$U(\boldsymbol{\theta}) = \frac{\partial}{\partial\theta}l(\boldsymbol{\theta}) = 0$$

i.e., for l=1,2...k

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\beta_l}} = \sum_{t=p+1}^{n}\frac{\partial l(\theta)}{\partial\mu_t}\frac{\partial\mu_t}{\partial\eta_t}\frac{\partial\eta_t}{\partial\boldsymbol{\beta_l}}$$

Note that, $\frac{\partial\mu_t}{\partial\eta_t} = \mu_t(1-\mu_t)$ and $\frac{\partial\eta_t}{\partial\boldsymbol{\beta_l}} = s_{tl} - \sum_{i=1}^{p}\phi_i s_{(t-i)l}$. Then,

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\beta_l}} = \sum_{t=p+1}^{N}(1-x_{ct})\zeta\,[log(\frac{x_t}{1-x_t}) -\{\psi(\mu_t\zeta) - \psi((1-\mu_t)\zeta)\}]\mu_t(1-\mu_t)(s_{tl} - \sum_{i=1}^{p}\phi_i s_{(t-i)l})$$

Here, let $x_t^* = log(\frac{x_t}{1-x_t})$ if $x_t\epsilon(0,1)$ else $x_t^* = 0$ and $\psi(\mu_t\zeta) - \psi((1-\mu_t)\zeta) = \mu_t^*$, where $\psi(.)$ is a digamma function. Then,

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\beta_l}} = \sum_{t=p+1}^{n}(1-x_{ct})\zeta(x_t^* - \mu_t^*)\mu_t(1-\mu_t)(s_{tl} - \sum_{i=1}^{p1}\phi_i s_{(t-i)l})$$

Similarly,

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\zeta}} = \sum_{t=p+1}^{n}\zeta(1-x_{ct})\{\mu_t(x_t^* - \mu_t^*) + log(1-x_t) -\psi((1-\mu_t)\zeta) + \psi(\zeta)\}$$

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\omega}} = \sum_{t=p+1}^{n}\frac{(x_{ct}-\omega)}{\omega(1-\omega)}$$

TABLE 1 Simulation results.

Model	Sample size (n)	100		250		500	
	Estimators	Bias	$\sqrt{MSE}$	Bias	$\sqrt{MSE}$	Bias	$\sqrt{MSE}$
	β_0	0.001	0.057	0.0006	0.0364	0.0001	0.026
DβAR(1)	ϕ_1	−0.002	0.072	−0.001	0.045	−0.0007	0.031
	ζ	−3.222	12.603	−1.308	6.883	−0.637	4.679
	ω	0.003	0.206	−0.0004	0.032	−0.00007	0.022
	β_0	0.005	0.110	0.0004	0.048	0.0008	0.034
	ϕ_1	−0.002	0.125	−0.0006	0.053	−3.77E−05	0.037
DβAR(2)	ϕ_2	-0.008	0.134	−0.002	0.055	-0.001	0.039
	ζ	−10.306	26.772	−1.758	7.159	−0.881	4.712
	ω	0.0003	0.073	−0.0003	0.032	−0.0001	0.022

For i=1,2...p

$$\frac{\partial l(\boldsymbol{\theta})}{\partial \boldsymbol{\phi_i}} = \sum_{t=p+1}^{n} (1 - x_{ct})\zeta_t(x_t^* - \mu_t^*)\mu_t(1-\mu_t)(g(x_{t-i}) - s'_{t-i}\boldsymbol{\beta})$$

The maximum likelihood estimator of $\boldsymbol{\theta}$ is obtained by equating $U(\boldsymbol{\theta}) = 0$. Since there exists no closed form solution for these equations, a non-linear optimization algorithm like Newton's method or a Quasi-Newton algorithm such as limited-memory Broyden–Fletcher–Goldfarb–Shanno algorithm (L-BFGS-B) has been used (14, 15).

Practically, we can use the gamlss function in R software in the package GAMLSS (generalized additive models for location scale and shape) (16) to get the initial values for estimating the parameters.

In this study, we have used the Quasi-Newton method (17) under which we performed the L-BFGS-B algorithm to obtain the optimum solution for the parameters.

Large sample inference: If the model specified by Equation (5) follows the regularity condition of maximum likelihood estimation (MLE) then, MLEs of $\boldsymbol{\theta}$ and $J(\boldsymbol{\theta})$ (Fisher information matrix) are consistent. Assuming that $I(\boldsymbol{\theta}) = \lim_{n\to\infty}\{n^{-1}J(\boldsymbol{\theta})\}$ exists and is non-singular, we have $\sqrt{n}(\hat{\boldsymbol{\theta}} - \boldsymbol{\theta})$ converges in distribution to $N(0, I(\boldsymbol{\theta})^{-1})$.

2.3. Simulation study

In this simulation study, we featured finite-sample performance of the MLE. Due to time consumption, the simulated time-series data generated only for DeβAR with lag 1 and lag 2.

$$\eta_t = logit(\mu_t) = \beta_0 + \phi_1 g(x_{t-1})$$

$$\eta_t = logit(\mu_t) = \beta_0 + \phi_1 g(x_{t-1}) + \phi_2 g(x_{t-2})$$

Here, ζ and ω are constant for all observations. We took $\beta_0 = 1.2$, $\phi_1 = -0.8$, $\phi_2 = -0.2$, $\zeta = 50$, and $\omega = 0.5$ as true parameters.

The Monte Carlo simulation with 15,000 replications was carried out each with sample size n = 100, 250, and 500. Of the 15,000 replicants, 5,000 were the burn out. Parameter estimates were obtained as the convergent outcome of the remaining 10,000. The parameters are estimated by maximizing the log-likelihood function using the L-BFGS-B algorithm. The bias and root mean square error of the estimates are reported.

Table 1 represents the bias and root mean square error of the parameters. Here, we can observe that, as the sample size increases, the algorithm converged for all the samples. The mean of β_0, ϕ_1, ϕ_2, and ω are close to the true values or the initial values. Also, the root mean square errors of all the estimators are decreased as the size of the sample increases, as anticipated.

2.4. Forecast evaluation criteria

Selecting a proper model among several competing candidates is a hassle in a lot of time-series analysis. In many cases, to look for out-of-sample forecast accuracy, the MAPE ("Mean absolute percentage error") is used for model selection or comparison. However, when data are close to zero, other forecast measurement criteria can be used, such as MAE ("mean absolute error"), MSE ("mean square error"), RMSE ("root mean square error"), and ex post forecast error (18). In the case of time-series analysis, the ACF plot is one of the model selection criteria, and the AIC ("Akaike information criterion") and the BIC ("Bayesian information criterion") will be used for an in-sample accuracy check.

TABLE 2 Descriptive statistics for the data on rate of *E. coli* resistant to AMC and CSL.

Antibiotic	Sample size	Range	Mean	Std.Deviation	Skewnes	Min	Max	Median
AMC	108	0.593	0.703	0.165	−0.0024	0.407	1	0.702
CSL	108	0.4	0.15	0.10	0.40	0	0.4	0.14

2.5. R shiny application

R shiny is a website application available freely to build under R-studio. It is an interactive website application that requires no web development skills[1]. R shiny web app can be accessed at DeBAR.app[2].

3. Results

3.1. Exploratory data analysis

To understand the characteristics of the response series, the descriptive statistics for the data are represented in Table 2. Figure 1 represents the time-series plot of the bacteria *E. coli* isolated from blood cultures resistant to the antimicrobial agents AMC and CSL, where the sudden change between the years 2015 and 2016 is due to the improvisation in the testing method in April 2015, which involved a shift from manual testing method (i.e., Kirby Bauer disc diffusion method) to Vitek-2 automated method from the year 2015. This change point has been considered as a covariate in the model and represented as an indicator variable I_t. Figure 2 displays the histogram of the same. The proposed model is applicable to model stationary time-series data. The ACF plot will be used to identify the stationarity in the series. If non-stationarity exists, then significant deterministic elements can be added as a regressor variable in the model. As the current study data include change points in the series, stationarity cannot be identified through the ACF plot.

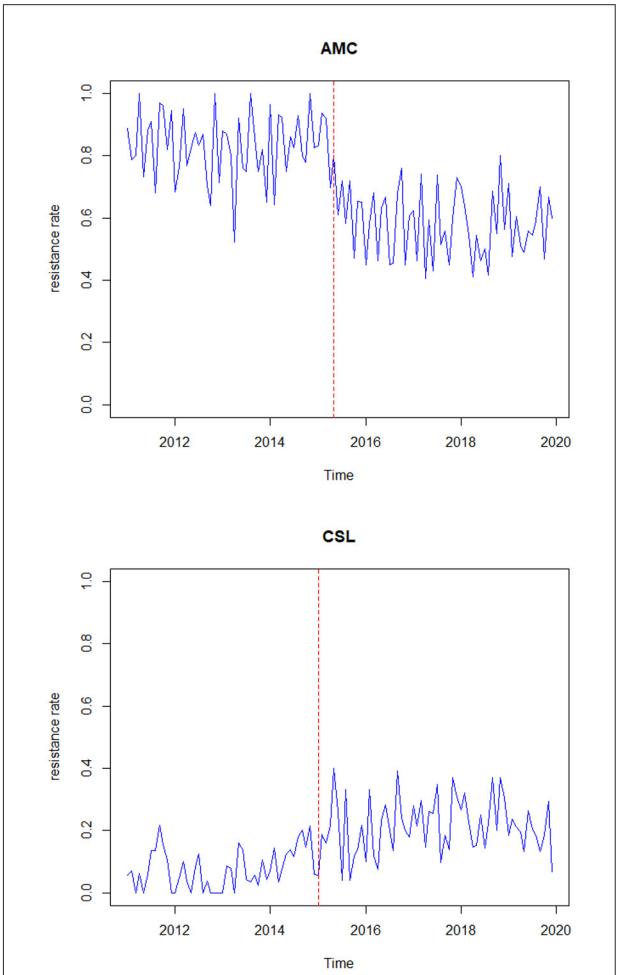

FIGURE 1
Time plot of proportion of *E. coli* in blood resistant to antibiotics AMC and CSL.

3.2. Time-series analysis

The data have been modeled using Degenerate Beta Autoregressive (DeβAR(p)) model proposed in Section 2 and the analysis has been carried out using the software R. Quasi-Newton algorithm (L-BFGS-B) has been used to maximize the partial log-likelihood function.

First, the following DeβAR(p) models, where p=1, 2, 3,...,12 fitted to the AMC data,

$$Model1: \eta_t = logit(\mu_t) = \beta_0 + \alpha I_t + \phi_1\{g(x_{t-1})\}$$

$$Model2: \eta_t = logit(\mu_t) = \beta_0 + \alpha I_t + \Sigma_{i=1}^{2}\phi_i\{g(x_{t-i})\}$$

$$Model3: \eta_t = logit(\mu_t) = \beta_0 + \alpha I_t + \Sigma_{i=1}^{3}\phi_i\{g(x_{t-i})\}$$

$$Model12: \eta_t = logit(\mu_t) = \beta_0 + \alpha I_t + \Sigma_{i=1}^{12}\phi_i\{g(x_{t-i})\}$$

where, x_{t-i} i=1,2,...,12 is the lagged response series and I_t is the change point (Indicator) variable with coefficient α.

1 https://shiny.rstudio.com/

2 https://jevithalobo.shinyapps.io/column/

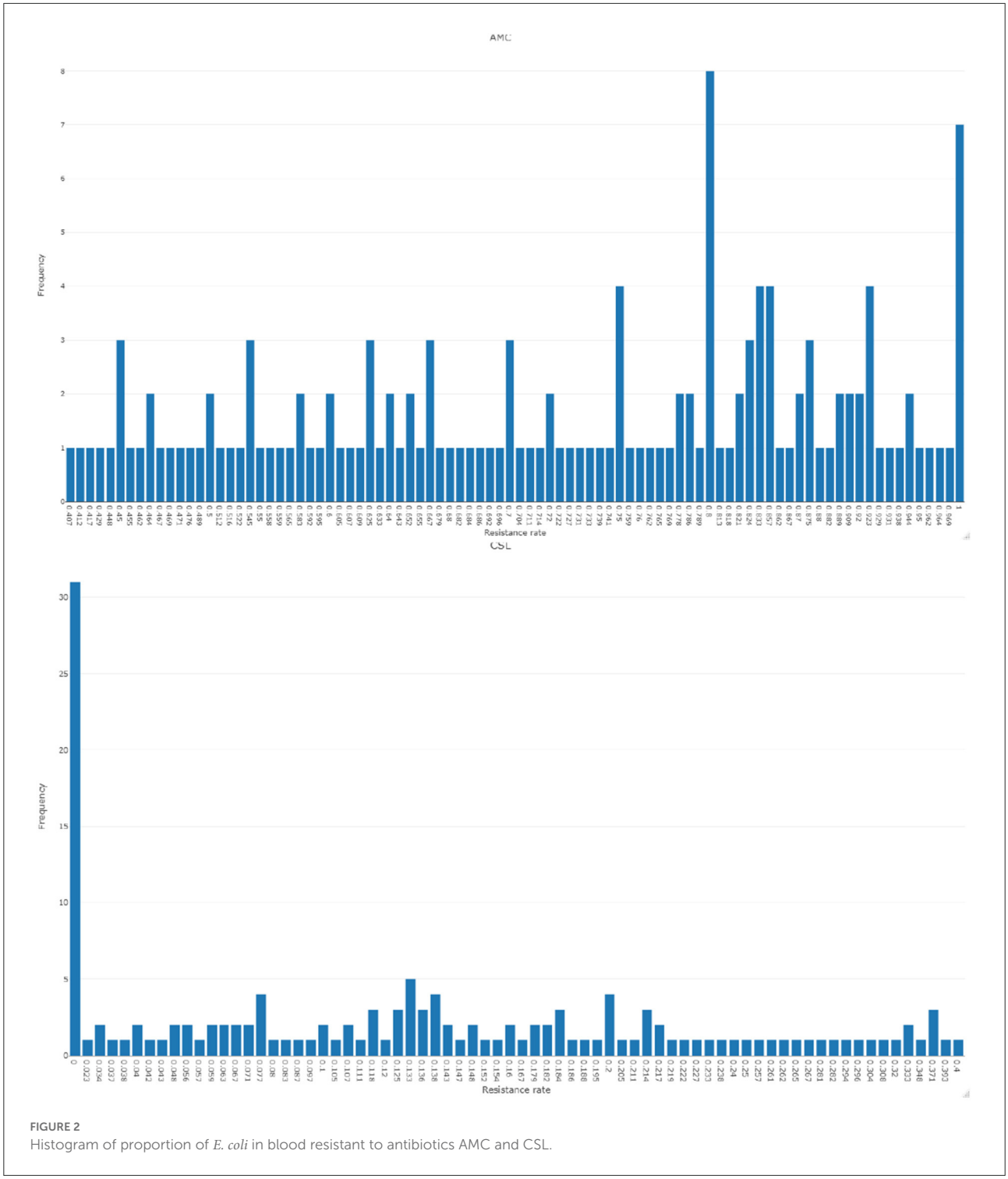

FIGURE 2
Histogram of proportion of *E. coli* in blood resistant to antibiotics AMC and CSL.

Among the 12 models, the best model is selected using the AIC and the BIC for which these values are minimum. The selected model is then compared with the existing ARIMA model.

The AIC and the BIC select Model 1 as the best among the 12 models as its values are minimum compared to the others (Table 4). From the significance test (Table 3), it can be seen that for the data AMC, lag-1 is significant at 5% level

TABLE 3 Fitted DeβAR model for rate of *E. coli* resistant to AMC and CSL.

Antibiotic	Parameter	Estimate	Std. Error	z stat	*p*-value
	β_0	1.689	0.156	10.808	$0.00e^{+00}$
	ϕ_1	−0.109	0.080	−1.367	$1.74e^{-02}$
AMC	$log(\rho)$	2.798	0.554	5.043	$2.15e^{-06}$
	$logit(\Omega)$	−3.188	0.099	−32.043	$0.00e^{+00}$
	α	−1.338	0.150	−8.889	$3.59e^{-14}$
	β_0	−2.185	0.166	−13.112	0.00e+00
	ϕ_1	−0.0215	0.068	−0.313	7.547e-02
CSL	$log(\rho)$	3.099	0.020	148.21	0.00e+00
	$logit(\Omega)$	−2.102	0.319	−6.580	2.46e-09
	α	0.877	0.128	6.834	7.55e-10

of significance (l.o.s) and for data CSL, lag-1 is significant at 10% level of significance (l.o.s) along with the indicator variable and parameters of the model, respectively. Next, the model is compared with the ARIMA model. Autocorrelation function (ACF) plots for residuals, forecast accuracy criteria (MAE, MSE, and MAPE), and information criteria (AIC and BIC) were used to select the best model for the given data.

Models considered were,

Model 1: DeβAR(p), with lag at 1

$$\eta_t = logit(\mu_t) = \beta_0 + \alpha I_t + \phi_1 g(x_{t-1})$$

Model 2: ARIMA(0, 0, 1) model using Arcsine transformation

$$y_t = \beta_0 + \alpha I_t - \theta_1 \epsilon_{t-1}$$

In the analysis, as a final step, data have been forecasted by keeping out the last six observations (hold-out data) from the time-series, and Model 1 and Model 2 have fitted to the selected (test data) series and forecasted for the next 6 months. The out-of-sample forecast accuracy are calculated using the hold-out and forecasted data from the models and comparisons between models have been carried out. The hold-out series of AMC is 0.545, 0.592, 0.7, 0.469, 0.667, and 0.6. The forecasted series from Model 1 is 0.597, 0.594, 0.6, 0.595, 0.595, and 0.6. The forecasted series from Model 2 is 0.602, 0.579,0.579, 0.579, 0.579, and 0.579. Similarly, the hold-out series of CSL is 0.205, 0.184, 0.133, 0.184, 0.294, and 0.067. The forecasted series from Model 1 is 0.192, 0.194, 0.193, 0.193, 0.194, and 0.195. The forecasted series from Model 2 is 0.223, 0.215, 0.213, 0.214, 0.214, and 0.213.

The study found that for both the data, the forecast accuracy for Model 1 is better compared to the Model 2 (Table 5) and residual plot of Model 1 follows white noise assumptions (i.e., the residual series should have mean 0 and no autocorrelation within the series), whereas for AMC data (Figure 3), Model 2 is serially correlated at lag 4. Thus, we select Model 1 as the best fit model for both the data.

Thus, the estimated DeβAR(1) model for AMC is

$$\hat{\mu_t} = \frac{exp(\beta_0 + \alpha I_t + \phi_1 g(x_{t-1}))}{1 + exp(\beta_0 + \alpha I_t + \phi_1 g(x_{t-1}))}$$

$$E(x) = \hat{\omega} + (1 - \hat{\omega})\hat{\mu_t}$$

where, $(\hat{\beta_0}, \hat{\alpha}, \hat{\phi_1}, \hat{\omega})$ = (1.68, −1.34, −0.11, 0.03). The forecasted series for next 6 months is 0.604, 0.587, 0.613, 0.587, 0.604, and 0.582.

Similarly, the estimated DeβAR(1) model for CSL is

$$\hat{\mu_t} = \frac{exp(\beta_0 + \alpha I_t + \phi_1 g(x_{t-1}))}{1 + exp(\beta_0 + \alpha I_t + \phi_1 g(x_{t-1}))}$$

$$E(x) = (1 - \hat{\omega})\hat{\mu_t}$$

where, $(\hat{\beta_0}, \hat{\alpha}, \hat{\phi_1}, \hat{\omega})$ = (−2.185,0.877, −0.021, 0.108). The forecasted series for next 6 months is 0.19, 0.20, 0.19, 0.194, 0.192, and 0.189.

The R code of the analysis has been provided in the supporting file.

4. Discussion

AMR is difficult to control and expensive to deal with, and the outcome is that it can lead to death or severe disability. Many researchers have used different statistical techniques to look at the relationship between antimicrobial use and resistance. To list a few of them, a study by Athanasiou and Kopsini (19) in 2018 systematically reviewed the statistical methods used to analyze the AMR rate time-series data. Many of the studies

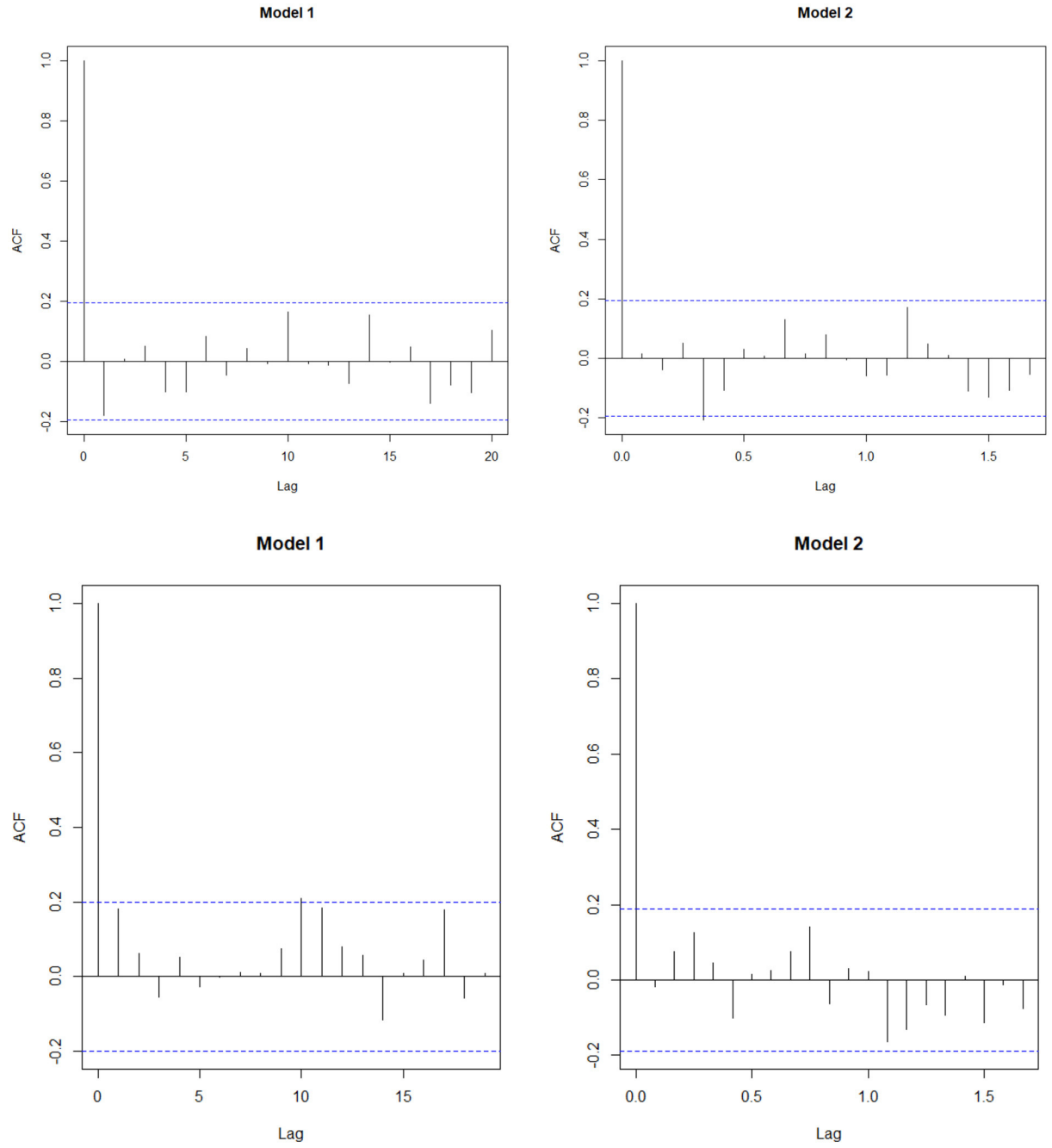

FIGURE 3
Sample ACF of the residuals obtained from the fitted models for AMC and CSL.

used the ARIMA model (20) and the transfer function model and few studies used the multiple linear regression model. Infectious disease control researchers most often analyze and predict antimicrobial resistance rate data, which includes zeros or ones [resistance rate with zeros can be seen in the figure of the article by Lopez-Lozano et al. (21)]. The commonly used ARIMA model is inappropriate for non-Gaussian (not normally distributed) time-series data and may arrive at biased results. Rocha and Cribari-Neto (7) and Bayer et al. (8) introduced the Beta autoregressive moving average model and the Kumaraswamy autoregressive moving average model to fit rate/proportion time-series data in the interval (0, 1). To analyze proportion data with zeros or ones, Ospina and Ferrari (11) proposed an inflated beta regression model, and as an

TABLE 4 AIC and BIC for DβAR(p) models for AMC and CSL data.

Antibiotic	AMC		CSL	
Model	AIC	BIC	AIC	BIC
Model 1	−126.19	−112.78	−153.69	−140.28
Model 2	−121.92	−105.83	−149.96	−133.86
Model 3	−117.64	−98.86	−152.58	−133.81
Model 4	−122.33	−100.87	−147.01	−125.55
Model 5	−119.55	−95.42	−151.37	−127.23
Model 6	−115.17	−88.35	−146.66	−119.84
Model 7	−110.99	−81.48	−142.14	−112.63
Model 8	−108.40	−76.22	-140.54	−108.35
Model 9	−105.93	−71.07	-138.07	−103.21
Model 10	−110.76	−73.22	−134.39	−96.84
Model 11	−106.62	−66.39	−133.56	−93.32
Model 12	−103.46	−60.55	−136.87	−93.96

TABLE 5 Model selection criterion.

Antibiotic	Model	Log-likelihood	MAE	MSE	MAPE	AIC	BIC
AMC	Model 1	68.09	0.089	0.011	13.93	−126.19	−112.78
	Model 2	57.81	0.109	0.018	18.40	−107.62	−97.12
CSL	Model 1	75.64	0.053	0.005	—	−153.69	−140.28
	Model 2	63.39	0.064	0.006	—	−118.79	−108.29

alternative, Bayer et al. (12) proposed an inflated Kumaraswamy regression model. But none of the studies addressed the autocorrelation in the response series in the interval [0, 1) or (0, 1]. So, to fill this gap, in this article, we proposed the Degenerate Beta Autoregressive (DeβAR) model.

For this intention, we collected time-series data on *E. coli* isolates resistant to the different antimicrobial agents, and for the current study, only amoxicillin–clavulanic acid (AMC) and Cefoperazone–sulbactam (CSL) were considered. The primary focus of this study is to forecast the AMR rate for the time-series data in the interval (0, 1] or [0, 1). The proposed DeβAR(p) models (where p=1,2,...,12) fitted to the data and best order for "p" was selected for which the values of the AIC and the BIC were minimum. The best model among these was then compared with the existing ARIMA model and the best among both was decided based on the forecasting evaluation criteria (MSE, MAE, and MAPE).

From Table 4, we can see that for the data AMC, the values of the AIC and the BIC are minimum for Model 1 (i.e., AIC = −126.19 and BIC = −112.78) compared to other models. The study found that the DeβAR (1) model performed well compared to the remaining 11 models. Next, the selected DeβAR(1) model is compared with the existing ARIMA (0, 0, 1) model selected from the autogeneration. From Table 5, we can see the values of MAE, MSE, and MAPE are minimum for the proposed Model 1 compared to the existing Model 2 along with the AIC and BIC. Hence, the study selected the DeβAR(1) model as the best among all. Similar procedure followed for the data of CSL.

By using the proposed DeβAR(1) model, the AMR rate was forecasted for next 6 months. The study results indicate the forecasted resistance rate of *E. coli* to the antimicrobial AMC ranges between 58 and 62% for the next 6 months, implying constant variations in the resistance rate.

The DeβAR(p) model introduced in this article would be beneficial to healthcare providers to implement early public health measures to control and prevent the rise in resistance rate.

5. Conclusion and future direction

Antimicrobial resistance is an emerging issue of public health. However, taking appropriate precautions or interventions in advance may help in reducing the resistance rate. Forecasting using an appropriate time-series model may

help in predicting the expected resistance rate for the future which is further useful for policy-making.

This study proposed the Degenerate Beta Autoregressive model (DeβAR) to model antimicrobial resistance rate data, which is an extension of the βARMA model proposed by Rocha and Cribabri-Neto (2009). The proposed model can be used to fit continuous time-series data in the interval [0, 1) and (0, 1], for example, rates or proportions. The model is applicable when the series is stationary in nature. The parameters of the model are estimated by maximizing the likelihood function and closed-form solutions for the score function are obtained by using the non-linear optimization algorithm (L-BFGS-B). The application of the model is presented using AMR data.

The outcome from a time-series model helps the healthcare policymakers to implement an appropriate intervention in advance to reduce the risk of rise in resistance rate.

In future, the study results can be improvised by considering antimicrobial consumption as a regressor variable and the proposed model can be improvised by incorporating moving average terms and seasonal components.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional Ethical Clearance was obtained from Kasturba Medical College and Kasturba Hospital Institutional Ethics Committee (IEC No. 832/2019). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

JL, AK, and VK framed the objective of the study. VK collected the data. JL conducted the analysis, drafted initial manuscript, and developed R shiny app. AK and VK revised the manuscript. All authors have read and approved the final manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.969777/full#supplementary-material

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The under investigated facet of the COVID-19 pandemic: Molecular analysis of secondary bacterial infections at a COVID dedicated intensive care unit within a tertiary care center in Lebanon

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Background: The coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has spread worldwide. Secondary bacterial infections are associated with unfavorable outcomes in respiratory viral infections. This study aimed at determining the prevalence of secondary bacterial infections in COVID-19 patients admitted at a tertiary medical center in Lebanon.

Methodology: From May till November, 2020, a total of 26 Gram-negative isolates were recovered from 16 patients during the course of their COVID-19 infection with *Escherichia coli* being the most prevalent. The isolates were assessed for their antimicrobial susceptibility by broth microdilution against 19 antimicrobial agents from different classes. Whole genome sequencing of 13 isolates allowed the mining of antimicrobial resistance (AMR) determinants as well as mobile genetic elements and sequence types (ST). Finally, broth microdilution with three different efflux pump inhibitors [theobromine, conessine and PheArg-β-naphthylamide (PAβ N)] was done.

Results: Antimicrobial susceptibility testing showed that out of the 26 Gram-negative isolates, 1 (4%) was extensively drug resistant and 14 (54%) were multi-drug resistant (MDR). Whole genome sequencing results revealed a plethora of AMR determinants among the 13 sequenced isolates. Moreover, the 9 Enterobacterales and 4 *Pseudomonas aeruginosa* sequenced isolates belonged to 9 and 2 different ST, respectively. Using a variety of efflux pump inhibitors we demonstrated that

only PAβN had a significant effect when combined with levofloxacin, and the latter regained its activity against two *P. aeruginosa* isolates.

Conclusion: The identification of carbapenem and colistin resistant Gram-negative bacilli causing secondary bacterial infections in critical patients diagnosed with COVID-19 should be of high concern. Additionally, it is crucial to monitor and track AMR, post-COVID pandemic, in order to better understand the effect of this disease on AMR exacerbation.

KEYWORDS

COVID-19, secondary bacterial infections, carbapenem resistance, colistin resistance, NDM-5, NDM-7, *mcr-1.26*

Introduction

A surprising cluster of pneumonia cases of unknown source arose in Wuhan, Hubei province, China in early December 2019. Some of the infected patients developed serious complications such as acute respiratory distress syndrome (ARDS) (1). January 2020, marked the identification of the disease as coronavirus disease 2019 (COVID-19) caused by a novel coronavirus, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (2). In March 2020, after the drastic increase in the number of cases worldwide, the World Health Organization (WHO) declared COVID-19 as a global pandemic (3).

During the course of this pandemic, several studies were conducted to establish a diagnosis, treatment, and preventive measures for this disease. The general symptoms include fever, cough, apnea, and nausea, but the most serious complication that has been associated with COVID-19 was pneumonia. In addition to the respiratory system, this disease also affects the hepatic, gastrointestinal, and neurological systems (3).

The association of SARS-CoV-2 with other viruses, bacteria and fungi poses many challenges in diagnosing and treating patients with COVID-19 (4). Zhu et al. (5) found that bacterial coinfections were more prominent than viral or fungal coinfections. Influenza-associated bacterial coinfections were originally associated with a substantial increase in morbidity and mortality during pandemics (6). Even though, there is still no clear evidence on how the bacterial coinfection is affecting the outcome of the viral disease, the majority of the studies show that it can aggravate its outcome (7). In this context and the rise in antimicrobial resistance (AMR), it is necessary to conduct investigations regarding bacterial coinfections in COVID-19 patients. Therefore, this study aimed at determining the prevalence of secondary Gram-negative bacterial infections in COVID-19 patients admitted to the intensive care unit (ICU) at a tertiary medical center in Lebanon during a specific time period. This study also investigates the molecular mechanisms of AMR within these bacterial pathogens.

Materials and methods

Bacterial isolates

Between May and November 2020, 137 patients were admitted to the medical center as COVID-19 patients. A total of 37 patients were admitted to the ICU and 16 out of the 37 patients developed bacterial co-infections due to Gram-negative pathogens. During that period of time, no other co-infections were recorded. A total of 26 Gram-negative isolates were recovered from 16 patients during the course of their COVID-19 infection admitted to the ICU at a tertiary medical center in Lebanon. They were distributed as: 8 *Escherichia coli*, 6 *Pseudomonas aeruginosa*, 4 *Enterobacter cloacae*, 3 *Klebsiella pneumoniae*, 2 *Stenotrophomonas maltophilia*, 1 *Klebsiella oxytoca*, *Providencia stuartii*, and *Acinetobacter baumannii* **Supplementary Table 1**. These consecutive isolates were recovered as part of the routine bacteriology workflow in the Pathology and Laboratory Medicine Department at the American University of Beirut Medical Center.

Antimicrobial susceptibility testing

Antimicrobial susceptibly testing was performed by broth microdilution against 19 different antimicrobial agents from different classes. Serial dilution was performed with concentrations ranging from 1,024 to 1 μg/ml and the plate was incubated at 37°C for 18–24 h. All the experiments were run in duplicates. The results were interpreted according to the CLSI M100 guidelines (8). Control strains *K. pneumoniae* ATCC® 13883, *E. coli* ATCC® 25922 and *P. aeruginosa* ATCC® 27853 were used in parallel to monitor the minimal inhibitory concentrations (MIC) results.

Whole genome sequencing

Based on the resistance profiles, a total of 13 Gram-negative isolates were selected for sequencing, distributed as: 6 *E. coli*, 4 *P. aeruginosa*, 2 *K. pneumoniae*, and 1 *E. cloacae*. These isolates were either MDR or extensively drug resistant (XDR). Isolates that fall outside these categories were not sequenced. To prepare whole-genome sequencing libraries, fresh cultures were grown on MacConkey agar and genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Sequencing libraries were prepared using the Nextera XT library prep kit (Illumina GmbH, Munich, Germany) and sequenced on the Illumina MiSeq sequencer, 2 × 150 bp. Reads quality control and trimming was done using Trimmomatic (v.1.2.14) after which assembly of the genome was

performed using Unicycler on Galaxy.[1] Sequences were deposited in NCBI under the BioProject number PRJNA613441.

Bioinformatic analysis

Antimicrobial resistance genes were acquired through ResFinder on Center of Genomic Epidemiology (CGE)[2] and CARD.[3] Plasmids harbored in each isolate were determined using PlasmidFinder on CGE.[4] Sequence types (ST) were identified using MLST on CGE.[5]

Efflux pump inhibitor assay

Following the analysis of the whole genome sequencing results, the presence of the *acrAB-TolC* and *MexAB-OprM* genes was investigated. The efflux pump inhibitor theobromine was used against *E. coli* isolates that harbored the *acrAB-TolC* gene and were resistant to ciprofloxacin and/or tetracycline. Moreover, the efflux pump inhibitors, conessine and PheArg-β-naphthylamide (PAβN), were used against *P. aeruginosa* isolates that harbored the *MexAB-OprM* and were resistant to levofloxacin. Their MIC were determined for both antibiotics with and without the selected efflux pump inhibitor. Antimicrobial/efflux pump inhibitor combinations experiment was performed by adding fixed concentrations of the inhibitor to the experimental wells of a standard broth microdilution assay. We followed CLSI guidelines in this assay. However, minor modifications to broth volumes were made in order to accommodate the presence of the efflux pump inhibitor while keeping the concentrations of the antimicrobials and the bacterial suspensions in accordance with CLSI recommendations. For the selected isolates, theobromine, conessine, and PAβN efflux pump inhibitors were used at a fixed concentration of 100 (9), 20, and 25 μg/ml (10), respectively. The MICs of the tested isolates were interpreted according to the CLSI M100 guideline (8).

Data analysis

IBM SPSS version 20 was used to perform data analysis. Descriptive statistics for all variables was used to analyze the data. Fisher's exact test and independent *t*-test were used to examine the association between baseline characteristics variables and mortality of COVID-19 patients after bacterial co-infection. The significance was set at $p < 0.05$.

IRB approval

IRB approval for this work was awarded by the American University of Beirut Institutional Review Board under the IRB ID BIO-2020-0541.

1 https://usegalaxy.org
2 https://cge.food.dtu.dk/services/ResFinder/
3 https://card.mcmaster.ca/
4 https://cge.food.dtu.dk/services/PlasmidFinder/
5 https://cge.food.dtu.dk/services/MLST/

Results

Baseline characteristics of COVID-19 patients after bacterial co-infection

Baseline characteristics are presented in Table 1. Sixty percentage of patients were males and the average age was 62.13 ± 21.05 years. All of the patients previously stayed in the ICU between 0 and 2 days within 30 days prior to admission to the COVID ICU. 14 (93.3%) patients had 0–2 days of mechanical ventilation use. 13 (87%) patients had 0–2 days prior hospital stay. Only 2 (13.3%) patients had diabetes, 3 (20%) patients had renal insufficiency, 3 (20%) patients had heart failure and 1 (6.7%) had malignancy. The baseline comorbidities are presented in Table 1.

The association between demographic characteristics and mortality rate

There was no statistically significant difference between baseline characteristics and mortality (Table 2).

The association between complications and mortality

Patients who developed sepsis ($p = 0.042$) or septic shock ($p = 0.044$) had a statistically significant higher rate of mortality

TABLE 1 Baseline characteristics of post-COVID-19 patients after bacterial co-infection.

Characteristic ($n = 15$)	No (%)
Male gender	9 (60%)
Age in years (mean, SD)	62.1 (21%)
Diabetes	2 (13%)
Renal insufficiency	3 (20%)
Gastrointestinal disease	1 (7%)
Malignancy	5 (33%)
Albumin in mg/dl (mean, SD)	32.8 (5%)
Hospital stay within 30 days	
0–2 days	13 (87%)
3–5 days	2 (13%)
>6 days	0 (0%)
Mechanical ventilation	
0–2 days	14 (93%)
3–5 days	0 (0%)
>6 days	1 (7%)
Hemodialysis	1 (7%)
Heart failure	3 (20%)
Steroids past 30 days	1 (7%)
Admission duration in days (mean, SD)	44.5 (60%)
Time at risk in days (mean, SD)	13.1 (19%)

TABLE 2 The association between demographic characteristics and mortality rate among COVID-19 patients after bacterial co-infection.

Characteristic (n = 15)	Outcome		
	Alive	Dead	P-value
Male gender	6 (66.7%)	3 (33.3%)	0.455
Age	58.3 ± 22.9	67.8 ± 18.3	0.278
Time at risk in days	14.6 ± 23.7	11.0 ± 9.9	0.367
Diabetes	2 (100%)	0	0.343
Renal insufficiency	2 (66.7%)	1 (33.3%)	0.659
GI diseases	1 (100%)	0	0.600
Malignancy	3 (60.0%)	2 (40.0%)	0.706
Albumin (mg/dl)	33.00 ± 3.92	32.50 ± 7.01	0.071
Hospital stay within 30 days			
0–2 days	9 (69.2%)	4 (30.8%)	0.143
3–5 days	0	2 (100%)	
Mechanical ventilation			
0–2 days	9 (64.3%)	5 (35.7%)	0.400
3–5 days	0	0	
> 6 days	0	1 (100%)	
Hemodialysis	1 (100%)	0	0.600
Heart failure	1 (33.3%)	2 (66.7%)	0.341
Steroids past 30 days	0	1 (100%)	0.400
Piperacillin/tazobactam use within 30 days of admission	2 (100%)	0	0.526
Carbapenems use within 30 days of admission	1 (25.0%)	3 (75.0%)	0.103
Fluoroquinolones use within 30 days of admission	2 (33.3%)	4 (66.7%)	0.131

(60%). However, the results of the other complications showed no significant difference in outcomes.

The association between therapy and mortality

11 (47.83%) patients were treated with combination therapy of antibiotics, out of which 6 (54.5%) were alive and 5 (45.5%) died. There was no statistically significant difference in outcomes between patients who took combination therapy and those who did not (p = 0.400). Furthermore, the average time to appropriate therapy was 0.95 ± 2.36 days for all the patients. The time to appropriate therapy for people who lived was 0.92 ± 2.14 days and for those who died was 1.00 ± 2.83 days. The difference between the two groups was not statistically significant (p = 0.994).

Antimicrobial susceptibility testing

Enterobacterales

A total of 17 Enterobacterales isolates were included in this study, distributed as: 8 *E. coli*, 4 *E. cloacae*, 3 *K. pneumoniae*, 1 *K. oxytoca* and *P. stuartii*. Broth microdilution results showed that out of the 17 isolates, 11 (65%) were resistant to cefuroxime, tetracycline, and trimethoprim/sulfamethoxazole, 10 (59%) to aztreonam and fosfomycin, 9 (53%) to ceftolozane/tazobactam, 8 (47%) to ceftazidime, ciprofloxacin, levofloxacin, azithromycin, tigecycline, and piperacillin/tazobactam, 7 (41%) to cefepime, 5 (29%) to imipenem and ertapenem, 4 (24%) to meropenem, and colistin, and 3 (18%) to gentamicin. Moreover, all the isolates were susceptible to amikacin (Table 3 and Supplementary Tables 4, 5).

Pseudomonas aeruginosa

Broth microdilution results showed that out of the 6 *P. aeruginosa* isolates, 4 (67%) were resistant to meropenem, imipenem, and piperacillin/tazobactam, 3 (50%) to cefepime, ciprofloxacin, levofloxacin, and ceftolozane/tazobactam, 2 (33%) to aztreonam, and 1 (17%) to ceftazidime. Furthermore, all the isolates were susceptible to gentamicin, amikacin, and colistin (Table 4 and Supplementary Table 6).

Stenotrophomonas maltophilia

Broth microdilution results of the 2 *S. maltophilia* isolates showed that both isolates were resistant to trimethoprim/sulfamethoxazole. Moreover, both were susceptible to ceftazidime and levofloxacin (Supplementary Table 8).

Acinetobacter baumannii

Broth microdilution results showed that the *A. baumannii* isolate was susceptible to all the tested antimicrobial, including: meropenem, imipenem, ceftazidime, cefepime, ciprofloxacin, levofloxacin, colistin, amikacin, gentamicin, tetracycline, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole (Supplementary Table 7).

Whole genome sequencing

A total of 13 isolates were sequenced, distributed as: 6 *E. coli*, 4 *P. aeruginosa*, 2 *K. pneumoniae*, and 1 *E. cloacae*.

Enterobacterales

Whole genome sequencing results showed that the 9 Enterobacterales isolates belonged to 9 different ST. The 6 *E. coli* isolates belonged to ST69, ST131, ST167, ST617, ST624, and ST648. Moreover, the 2 *K. pneumoniae* isolates belonged to ST45 and ST307, and the *E. cloacae* isolate belonged to ST732.

A total of 134 AMR determinants were detected among the 9 sequenced Enterobacterales isolates. They encoded resistance to various antimicrobial families, such as: β-lactams, fluoroquinolones, aminoglycosides, and colistin. Several AMR determinants that encode resistance for β-lactams were detected, some examples include $bla_{CTX-M-15}$ (n = 6), bla_{TEM-1B} (n = 6), bla_{SHV-26} (n = 1), $bla_{CMY-145}$ (n = 1), bla_{OXA-1} (n = 5), bla_{NDM-5} (n = 3), and bla_{NDM-7} (n = 1). Moreover, those that encode resistance for aminoglycosides include *aph(3″)-Ib* (n = 7), *aac(6′)-Ib-cr* (n = 5), and *aadA* (n = 2). *FosA* (n = 3), *FosA3* (n = 2), and *FosA6* (n = 2) are some of the AMR determinants that encoded resistance against fosfomycin. Colistin resistance was due to the *mcr-1.26* gene in one *E. coli* isolates and mutation in the *parC* (n = 6) and *gyrA* (n = 6) in addition to other AMR determinants encoded resistance to fluoroquinolones. Resistance to disinfectants was encoded by *qacE* (n = 6), *sitABCD* (n = 4), *OqxA* (n = 2), and *OqxB* (n = 2) determinants (Supplementary Table 2).

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TABLE 3 Antimicrobial susceptibility testing results of the 17 Enterobacterales isolates.

Antimicrobials	No. resistant (%)					
	E. coli (*n* = 8)	*E. cloacae* (*n* = 4)	*K. pneumoniae* (*n* = 3)	*K. oxytoca* (*n* = 1)	*P. stuartii* (*n* = 1)	Total (*n* = 17)
Meropenem	3	1	0	0	0	4 (24%)
Imipenem	3	1	0	0	1	5 (29%)
Ertapenem	3	1	1	0	0	5 (29%)
Cefuroxime	7	1	3	0	0	11 (65%)
Ceftazidime	5	1	2	0	0	8 (47%)
Cefepime	5	1	1	0	0	7 (41%)
Aztreonam	7	1	2	0	0	10 (59%)
Ciprofloxacin	6	1	1	0	0	8 (47%)
Levofloxacin	6	1	1	0	0	8 (47%)
Colistin	1	1	1	0	1	4 (24%)
Amikacin	0	0	0	0	0	0 (0%)
Gentamicin	0	1	2	0	0	3 (18%)
Fosfomycin	2	3	3	1	1	10 (59%)
Azithromycin	5	1	1	0	1	8 (47%)
Tetracycline	6	1	3	0	1	11 (65%)
Tigecycline	2	2	3	0	1	8 (47%)
Trimethoprim/sulfamethoxazole	7	1	3	0	0	11 (65%)
Piperacillin/tazobactam	4	1	3	0	0	8 (47%)
Ceftolozane/tazobactam	5	1	3	0	0	9 (53%)

A total of 27 plasmids were harbored among the 6 *E. coli* isolates, distributed as: IncFIB(AP001918) (*n* = 5), IncFIA (*n* = 4), IncFII (*n* = 4), Col(BS512) (*n* = 3), IncFIB(pB171) (*n* = 1), IncFIC(FII) (*n* = 1), IncI(Gamma) (*n* = 1), IncFIA (*n* = 1), IncN (*n* = 1), IncX4 (*n* = 1), IncQ1 (*n* = 1), Col(MG828) (*n* = 1), Col156 (*n* = 1), Col440I (*n* = 1), and p0111 (*n* = 1). Moreover, the 2 *K. pneumoniae* isolates harbored 3 plasmids, IncFIB(K) (*n* = 2), IncFII(K) (*n* = 2), and Col(pHAD28) (*n* = 1). The *E. cloacae* isolate harbored the IncHI2, IncHI2A, and IncX3 plasmids.

TABLE 4 Antimicrobial susceptibility testing results of the 6 *P. aeruginosa* isolates.

Antibiotics	No. resistant (%)
Meropenem	4 (67%)
Imipenem	4 (67%)
Ceftazidime	1 (17%)
Cefepime	3 (50%)
Aztreonam	2 (33%)
Ciprofloxacin	3 (50%)
Levofloxacin	3 (50%)
Colistin	0%
Gentamicin	0%
Amikacin	0%
Piperacillin/tazobactam	4 (67%)
Ceftolozane/tazobactam	3 (50%)

Pseudomonas aeruginosa

Whole genome sequencing results showed that the 4 *P. aeruginosa* isolates belonged to 2 different ST, distributed as: ST309 (*n* = 3) and ST111 (*n* = 1).

A total of 67 AMR determinants were detected among the 4 sequenced *P. aeruginosa* isolates. They encoded resistance to various antimicrobial families, such as: β-lactams, fluoroquinolones, and aminoglycosides. Resistance to β-lactams was encoded by several AMR determinants, including bla_{OXA-50} (*n* = 3), $bla_{OXA-395}$ (*n* = 1), and $bla_{OXA-846}$ (*n* = 3). *crpP* (*n* = 1) and mutation in the *gyrA* (*n* = 2) led to fluoroquinolones resistance. Fosfomycin resistance was encoded by *FosA* (*n* = 4) and that against aminoglycoside included *emrE* (*n* = 4), *aph(3′)-IIb* (*n* = 4), and *aac(6′)-29b* (*n* = 1) determinants. *Sul1* (*n* = 1) and *qacE* (*n* = 1) encoded resistance against sulphonamides and disinfectants, respectively, **Supplementary Table 3**.

Efflux pumps inhibitors

Enterobacterales

Theobromine is an efflux pump inhibitor that inhibits the efflux of ciprofloxacin and tetracycline encoded by *acrAB-TolC* gene. Sequencing results showed that the latter was detected in 6 *E. coli* isolates out of the 9 sequenced Enterobacterales ones. Broth microdilution results, with and without theobromine, against ciprofloxacin and tetracycline showed that there was no change in the MIC of both antimicrobials in the presence of theobromine (**Table 5**).

Pseudomonas aeruginosa

Conessine and PAβN are efflux pump inhibitor that inhibits the efflux of levofloxacin encoded by *MexAB-OprM* gene in *P. aeruginosa*. Sequencing results showed that the latter was harbored in all the 4 sequenced *P. aeruginosa* isolates. However, this experiment was just done on the levofloxacin resistant (n = 3). Broth microdilution results, with and without conessine, showed that there was no change in the MIC of levofloxacin in the presence of conessine. However, the results in the presence and absence of PAβN showed a 16-fold decrease in the MIC of levofloxacin in 1 isolate, a 4-fold decrease in 1 isolate, and no change in MIC in 1 isolate (**Table 6**).

Discussion

The mortality and morbidity rates in viral infections increase when it is coupled with secondary bacterial infections, especially in case of viral ARDS (11). SARS-CoV-2 can facilitate the attachment and colonization of bacteria to the respiratory tissues of the host. Likewise, secondary bacterial infections can facilitate the systemic spread of the virus, increasing the risk of systemic infections and sepsis (12). Around 200 million viral community acquired pneumonias occur every year and various studies addressed the issue of secondary bacterial infections and viral pneumonia (13). In the case of COVID-19, studies done in several countries showed a variation in the prevalence of bacterial secondary infections among patients diagnosed with the virus, ranging between 1 and 50% (12).

In this study, a total of 26 Gram-negative isolates were recovered from 16 patients in the course of their COVID-19 infection. *E. coli* was the most recovered bacterial pathogen, followed by *P. aeruginosa*, *E. cloacae*, *K. pneumoniae*, *S. maltophilia*, *K. oxytoca*, *P. stuartii*, and *A. baumannii*. Although the most frequently isolated bacteria causing secondary bacterial infection in COVID-19 patients varied from one study to another (12, 14–16). All obtained Gram-negative bacilli isolates from COVID-19 patients in our study, such as *E. coli* (14), *P. aeruginosa* (15), *K. pneumoniae*, *K. oxytoca* (12), *E. cloacae* (2), *A. baumannii* (16), and *S. maltophilia* (17) match those isolated in similar studies in the literature.

TABLE 5 Broth microdilution results for ciprofloxacin and tetracycline, with and without theobromine, for 6 *E. coli* isolates.

	MIC (μg/ml) [Int]			
	Ciprofloxacin		Tetracycline	
Isolate code	Without theo	With theo	Without theo	With theo
E. coli 64	128 [R]	128 [R]	128 [R]	128 [R]
E. coli 65	64 [R]	64 [R]	256 [R]	256 [R]
E. coli 66	128 [R]	128 [R]	–	–
E. coli 67	16 [R]	16 [R]	256 [R]	256 [R]
E. coli 69	256 [R]	256 [R]	256 [R]	256 [R]
E. coli 71	–	–	64 [R]	64 [R]

Theo, theobromine; Int, interpretation; R, resistant.

TABLE 6 Broth microdilution results for levofloxacin, with and without conessine and PheArg-β-naphthylamide, for 3 *P. aeruginosa* isolates.

	MIC (μg/ml) [Int]		
	Levofloxacin		
Isolate code	Without EPI	With conessine	With PAβN
PSA35	2 [I]	2 [I]	2 [I]
PSA52	64 [R]	64 [R]	16 [R]
PSA61	32 [R]	32 [R]	2 [I]

PAβN, pheArg-β-naphthylamide; EPI, efflux pump inhibitor; R, resistant; I, intermediate; Int, interpretation.

Broth microdilution results showed that out of the 17 Enterobacterales isolates, 1 *E. cloacae* was XDR, 11 (7 *E. coli*, 3 *K. pneumoniae*, and 1 *P. stuartii*) were MDR, 2 (1 *E. coli* and 1 *E. cloacae*) were resistant to 2 antimicrobials, 2 (1 *K. oxytoca* and 1 *E. cloacae*) to 1 antimicrobial, and 1 *E. cloacae* isolate was susceptible to all tested antimicrobials. Six (3 *E. coli*, 1 *K. pneumoniae*, 1 *E. cloacae*, and 1 *P. stuartii*) out of the 17 Enterobacterales isolates were carbapenem resistant. Falcone et al. also described secondary bacterial infection caused by carbapenem resistant Enterobacterales in COVID-19 patients (18). Moreover, 3 (1 *E. coli*, 1 *K. pneumoniae*; and 1 *E. cloacae*) out of the 17 Enterobacterales isolates were colistin resistant (*P. stuartii* was not included in the count). Out of 6 *P. aeruginosa* isolates, 3 (50%) were MDR, 2 (33%) were resistant to 2 antimicrobials each, and 1 (17%) was susceptible to all the tested antimicrobials. Carbapenem resistance was detected in 4 (67%) out of the 6 *P. aeruginosa* isolates. Gysin et al. similarly identified carbapenem resistant *P. aeruginosa* isolates causing secondary bacterial infection in COVID-19 patients (19).

Whole genome sequencing results showed that all the 9 Enterobacterales isolates belonged to different STs. The 6 *E. coli* isolates belonged to ST69, ST131, ST167, ST617, ST624, and ST648. Moreover, the 2 *K. pneumoniae* isolates belonged to ST45 and ST307, and the *E. cloacae* isolate belonged to ST732. Moreover, the 4 *P. aeruginosa* were distributed into 2 different STs: ST309 (n = 3) and ST111 (n = 1). During the time of the study, we did not observe a nosocomial outbreak in the COVID-19 dedicated ICU wing. The reason behind that might be the fact that the COVID critical care unit and the COVID unit of non-critically ill patients were newly established in March 2020 in preparation for the pandemic. Both units were physically placed in a separate building from the main hospital and were newly furbished with the necessary equipment. Medical teams covering these two units did not take care of any other patient population. All healthcare teams in the units were provided with personnel protective equipment and the training necessary for its use.

A plethora of AMR determinants were detected among the sequenced isolates. Carbapenem resistance gene bla_{NDM-5} was detected in 3 *E. coli* isolates belonging to ST167, ST617, and ST648. In china, an *E. coli* isolate belonging to ST167 and harboring the bla_{NDM-5} gene was recovered from a patient at the teaching hospital of Zhengzhou University. *E. coli* isolates belonging to ST167 are classified as an internationally disseminated clonal lineage and they are associated with the global spread of $bla_{CTX-M-15}$ and bla_{NDM} genes in both humans and animals (20). The *E. coli* belonging to ST167 in our study harbored the $bla_{CTX-M-15}$ as well as the bla_{NDM-5} gene. Tian et al. described an *E. coli* isolate belonging to ST617 harboring the bla_{NDM-5} gene isolated from a patient admitted to a Children's Hospital in Shanghai, China (21). In 2019, an *E. coli* isolate recovered from a patient admitted to a Japanese university hospital was found to belong to ST648 and harbor the bla_{NDM-5} gene (22). Furthermore, in our study a bla_{NDM-7} gene was found to be

harbored in an *E. cloacae* isolate belonging to ST732. In addition to that, an *E. coli* belonging to ST624 and harboring the *mcr-1.26* gene was detected in our study. Poirel et al. (23) reported 2 *E. coli* isolates belonging to ST624 and harboring the *mcr-1* gene obtained from 2 patients in South Africa. Three out of the 4 sequenced *P. aeruginosa* isolates harbored the bla_{OXA-50} gene and belonged to ST309. In Switzerland, sequencing results of 8 *P. aeruginosa* isolates recovered from COVID-19 patients showed that they harbor the bla_{OXA-50} gene (19). Taking into consideration what is already known regarding the accuracy of whole genome sequencing (WGS) and its benefits, the identification when compared between the latter method and the conventional microbiology were in complete agreement in this study. However, the superiority of WGS was displayed in the amount of information, the level of details and the time required for the experiment. Using conventional techniques would have required days in order to gather such information where with WGS this was possible in less than 48 h.

Disinfectants were extensively used as sanitizers against COVID-19 throughout the pandemic. However, this may lead to major consequences in terms of AMR. In our study, 4 AMR determinants (*OqxA*, *OqxB*, *qacE*, and *sitABCD*) that encode resistance against disinfectants were detected. *OqxAB* efflux pumps are encoded by two *OqxA* and *OqxB* genes that are localized in one operon. Plasmid-encoded *OqxA* and *OqxB* efflux pumps can confer resistance to several antimicrobial agents, such as fluoroquinolones, chloramphenicol, and tigecycline (24, 25). The *qacE* gene encodes the efflux of antiseptics, mainly quaternary ammonium compounds, and they can be found on plasmids (26). The function of the *sitABCD* gene is to mediate the transport of Mn^{2+} and a Fe^{2+} transporter and it can be harbored on plasmids (27). Since all of these genes can be plasmid-borne, there dissemination due to the irrational use of disinfectants may pose a great risk on public health.

The *acrAB-TolC* efflux pump gene was detected in 6 sequenced *E. coli* isolates. Theobromine was used as an inhibitor for this gene (28). The efflux pump inhibitor assay results showed that no change in the MIC of both ciprofloxacin and tetracycline occurred once theobromine was added. This shows that the *acrAB-TolC* efflux pump gene was not expressed and was not involved in the resistance against both antimicrobials in the 6 *E. coli* isolates. Moreover, both conessine and PAβN, were used as inhibitors for the *MexAB-OprM* efflux pump gene in the 3 sequenced *P. aeruginosa* isolates (28). Although the MIC of levofloxacin against the 3 *P. aeruginosa* isolates did not change after the addition of conessine. A 16-fold decrease in the MIC of levofloxacin in 1 isolate, a 4-fold decrease in 1 isolate, and no change in MIC in 1 isolate was detected once PAβN was added. This may show that conessine did not inhibit the activity of the *MexAB-OprM* efflux pump gene. However, the inhibition of the latter by PAβN led to a decrease in the MIC of levofloxacin in 2 *P. aeruginosa* isolates but none became susceptible. This may be due to the detected mutations in the *gyrA* determinants in both isolates.

Conclusion

Further studies are required to investigate the association of comorbidities, complications during hospital stay, antimicrobial use and mortality in COVID-19 patients with secondary bacterial infections. Detection of carbapenem and colistin resistance in Gram-negative bacilli isolates causing bacterial secondary infection should be alarming. Accordingly, it is of high importance to monitor secondary bacterial infections in critical patients diagnosed with COVID-19. A limitation for this study was the inability to keep collecting more isolates and keep the surveillance active since the deadly wave in Lebanon began in December. A total lockdown took place in the country and hospitals became overwhelmed with dying patients. However, additional studies are needed to assess the impact of antimicrobial therapy on therapeutic outcome in COVID-19 patients to prevent antimicrobial overuse.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in this article/**Supplementary material**.

Author contributions

AS, PA, LH, and PM contributed toward the execution of the microbiology experiments. GDo and FA collected the clinical data. GA supplied the clinical isolates. IB, GDb, SK, GA, RM, and GM contributed in writing the grant and the manuscript. AA led the microbiology experimental section. ZK led the clinical section. All authors contributed to writing and editing the final draft of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2023.1001476/full#supplementary-material

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Differentiating methicillin resistant and susceptible *Staphylococcus aureus* from ocular infections using photoacoustic labeling

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Introduction: Antibiotic resistance in bacterial species constitutes a growing problem in the clinical management of infections. Not only does it limit therapeutic options, but application of ineffective antibiotics allows resistant species to progress prior to prescribing more effective treatment to patients. Methicillin resistance in *Staphylococcus aureus* is a major problem in clinical infections as it is the most common hospital acquired infection.

Methods: We developed a photoacoustic flow cytometer using engineered bacteriophage as probes for rapid determination of methicillin resistance in *Staphylococcus aureus* with thirteen clinical samples obtained from keratitis patients. This method irradiates cells under flow with 532 nm laser light and selectively generates acoustic waves in labeled bacterial cells, thus enabling detection and enumeration of them. *Staphylococcus aureus* isolates were classified from culture isolation as either methicillin resistant or susceptible using cefoxitin disk diffusion testing. The photoacoustic method enumerates bacterial cells before and after treatment with antibiotics. Decreasing counts of bacteria after treatment indicate susceptible strains. We quantified the bacterial cells in the treated and untreated samples.

Results: Using k-means clustering on the data, we achieved 100% concordance with the classification of *Staphylococcus aureus* resistance using culture.

Discussion: Photoacoustics can be used to differentiate methicillin resistant and susceptible strains of bacteria from ocular infections. This method may be generalized to other bacterial species using appropriate bacteriophages and testing for resistance using other antibiotics.

KEYWORDS

flow cytometry, optoacoustics, ultrasonic, k-means, microbial

1. Introduction

Methicillin resistant *Staphylococcus aureus* (*S. aureus*) (MRSA) infections are on the rise in both community and hospital settings (1, 2). Several studies have shown an increased rate of MRSA infections and that they account for the majority of clinically treated eye infections and they are the second most prevalent health care associated infection after *Pseudomonas aeruginosa* (3–5). Resistance to antibiotics is a natural process that has accelerated by human use of antibiotics for medicine and agriculture. In an effort to slow the rate of growth of antibiotic resistance, rapid identification and assessment of bacteria is essential so that antibiotics can be properly selected. New antibiotics have been slow to develop with only two completely novel antibiotics brought into use in the last 75 years (6). In the United States, an estimated $30 billion is spent annually on dealing with antibiotic resistant bacteria (7).

One group of bacteria is primarily responsible for the majority of multi-drug resistant infections. The ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter*) are responsible for the majority of nosocomial infections and are most likely to be multi-drug resistant (6). An estimated 90% of human antibiotic use is broad spectrum prescribed (7). From food processing plants to hospital beds, the speed at which bacterial contamination can be identified is the most important factor in treatment and control. Rapid bacterial identification negates the need for broad spectrum antibiotic use and allows for targeted therapy. Characterization of these pathogens not only requires their identification, but also determining their susceptibility to antibiotics. Ideally, rapid bacterial detection and identification of resistance would be fast enough to negate the use of broad spectrum antibiotics, therefore allowing point of care facilities to test and obviating the need for expensive equipment.

We have developed a photoacoustic method for detection of dilute particles in body fluids, *in vitro*(8, 9). Much of this work has been used for detecting circulating tumor cells (CTCs), primarily melanoma cells, as their native optical absorber, melanin, makes them suitable for sensitive photoacoustic detection and enumeration. This photoacoustic method is a type of flow cytometry in which cell suspensions are irradiated with nanosecond laser light that targets optically absorbing particles. These particles subsequently generate acoustic waves that are then detected using ultrasonic transducers. These signals are then counted, providing information about the number of particles. This number has been used to indicate disease state in cancer patients by determining the relative number of CTCs over time. Using bacteriophage that are engineered to absorb laser light, we specifically label bacterial cells in a sample and are able to perform the same type of photoacoustic flow cytometry (10–12). We showed that, like CTCs, we can detect single bacterial cells using this method.

For bacterial detection under flow, we need to induce optical absorption in these otherwise colorless cells. Bacteriophage viruses have evolved to have tail fibers that specifically recognize and bind to target bacterial cells. Figure 1 shows a DET7 bacteriophage that specifically binds to 60% of the 2,700 serovars of salmonella. If one of these serovars of salmonella is present among DET7 bacteriophage, DET7 will bind to the surface and eventually infect the cell by injecting the virus's own genetic material. We add a red protein dye to the bacteriophage so that after recognizing and binding to their target bacteria, we have essentially painted the bacterial cells with red dye, allowing us to target these cells with our laser and generate ultrasonic waves in our photoacoustic flow cytometer. Conceptually, for clinical testing to identify a bacterial infection in a patient, the infected blood sample will be split into several subsamples. A different type of bacteriophage will be added to each subsample. Only the subsample with the bacterial species that matches the bacteriophage will be painted with dye and, hence, will be the only subsample that results in photoacoustic detections. This is the basic method for rapid bacterial identification.

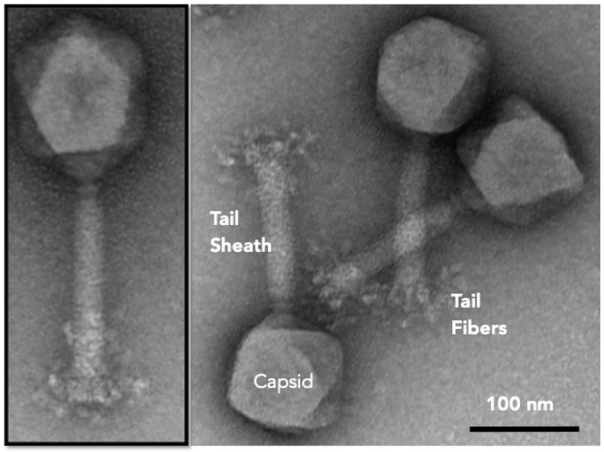

FIGURE 1
DET7 bacteriophage showing polyhedral capsid, tail sheath, and tail fibers with recognition proteins that attach to specific bacteria.

Once the infection is identified, a new blood sample can be split into two subsamples. Presumably, each subsample will contain approximately the same number of bacteria. One sample will be treated with an antibiotic agent while the other will be untreated. After two hours, both subsamples will be tested with the photoacoustic flow cytometer which will count the bacterial cells. If the number from both subsamples is approximately the same, we can assume that the bacteria is resistant to that antibiotic. If the treated subsample has significantly fewer cells, indicated by the number of photoacoustic events, then antibiotic susceptibility is indicated.

Competing technologies using polymerase chain reaction (PCR), fluorescence in situ hybridization (FISH), or bacterial culture require amplification, either in terms of genetic material or organism number (13–17). This requirement introduces significant delays in returning results and may introduce contamination. Moreover, both PCR or FISH involve analysis of molecular material, rather than the culprit bacteria themselves. Standard blood culture and PCR are the only methods in widespread clinical use. Though relatively inexpensive, blood cultures require an incubation period of 24–96 h, with the possibility of unsuccessful culturing or of culturing a competing bacterial species that is not the cause of pathology. The cost of PCR is highly variable,

on the order of $100, and still requires a culturing period, with the complication of choosing targeted reagents and amplifying unexpressed genes.

There are other research attempts for early detection of bacterial infection, including size, mechanical property, electrical, and acoustic types of classification (18–21). While these methods are innovative and exploit powerful techniques, they fundamentally work as enrichment tools, as these properties are all subject to high levels of biological variability. Consequently, discrimination must be done with higher sensitivity and lower specificity, so that most bacterial cells are selected, at the cost of excess blood cell contamination. The major shortcoming in all such methods is that they do not select for bacteria, but instead select for ranges of properties of bacterial cells that are shared with most bacterial cell types.

In this study, we obtained samples of *S. aureus* from patients who were treated for microbial keratitis. We tested each sample photoacoustically and determined whether these samples were methicillin resistant and compared our results to genetic testing for methicillin resistance.

2. Materials and methods

2.1. Photoacoustic system

Our photoacoustic system is shown in Figure 2 and consisted of a laser coupled to an optical fiber directed at a flow chamber through which saline suspensions were directed. An acoustic transducer was coupled to detection electronics for data processing and analysis. The system was calibrated using 10 μm dyed polystyrene microspheres (Polybead, Warrington, Pennsylvania) and phosphate buffered saline (Fisher Scientific, Pittsburgh, Pennsylvania) as positive and negative controls. As a control for bacteriophage binding, we used American Type Culture Collection strain 35556 (*S. aureus* strain SA113, ATCC, Manassas, Virginia). Modified bacteriophage SP1 was used as tags at a ratio of 1,000 bacteriophage per *S. aureus* cell. Bacteriophage were added to each culture and incubated at room temperature for 10 minutes to ensure phage binding. The combined culture and bacteriophage mixture was passed through the PAFC system at a flow rate of 60 μL/min.

The photoacoustic flow cytometer used a frequency doubled Nd:YAG laser operating at 532 nm with a 5 ns pulse duration and a 20 Hz pulse repetition rate. These laser parameters are appropriate for inducing acoustic waves in labeled bacteriophage attached to bacterial cells. Laser light was launched into a 1,000 μm optical fiber with a numerical aperture of 0.22 (Thorlabs, Newton, New Jersey). The optical fiber was directed to a flow chamber made from 3D printed polylactic acid (PLA) filament. The chamber is shown in Figure 3. An immersion acoustic transducer (Olympus, Waltham, Massachusetts) fixed to the flow chamber with a center frequency of 2.25 MHz and a focal length of 0.5 inches was used to sense the generated acoustic waves.

Rather than sending a continuous flow of cell suspension through the flow chamber, we induced two phase flow by introducing an immiscible fluid to the saline suspension. We used mineral oil, thus creating alternating droplets of cell suspension and oil (22, 23). These alternating droplets created a fluidic conveyor belt that allowed for localized detection of photoacoustic events. This arrangement allowed for microfluidic capture of droplets that generated photoacoustic waves which identified bacterial cells of interest.

The transducer was coupled to a high frequency digitizer and amplifier (National Instruments, Austin, Texas) connected to a desktop computer (Dell, Round Rock, Texas). Photoacoustic waves were identified by a LabVIEW (National Instruments, Austin, Texas) program made for this photoacoustic flow cytometer. Photoacoustic events were classified by a simple threshold of the voltage signal from the transducer. The threshold was set at three times the standard deviation of the noise. Each photoacoustic wave was assumed to be generated from a single bacterial cell, which was reasonable from the dilute concentration of bacterial cells. The bacterial count was recorded for each patient sample which as split into two subsamples, one of which was treated with oxacillin, and one was untreated. These numbers were used for determination of antibiotic resistance.

For quality control, we calibrated the photoacoustic system before each use. We ran a sample of phosphate buffered saline (PBS) as a negative control. In all PBS samples, we detected no photoacoustic events, as expected, as there were no optical absorbers present. For a positive control, we ran a suspension of 1 μmblack latex microspheres to ensure we were successfully detecting photoacoustic events. In all such cases, we showed constant detections, as the microspheres generated photoacoustic waves.

2.2. Sample preparation

S. aureus samples were obtained from The Charles T. Campbell Eye Microbiology Laboratory, University of Pittsburgh Medical Center, Pittsburgh, Pennsylvania, USA. A total of 13 samples were tested for the methicillin resistance using disk diffusion (24), thus determining their methicillin resistance before photoacoustic testing. Streaks of each *S. aureus* strain were grown on mannitol salt agar plates. Single colonies from each streak plate were used to regrow strains in mannitol salt broth for 2 h in a shaking water bath at 36.5 °C. This period ensured cells were growing and entering exponential growth phase. Oxacillin was added at a final concentration of 1 μg/ml to half of each culture and grown for an additional 2 h (25). We cultured on agar plates solely for comparison to plate reader results. The photoacoustic method, in clinical implementation, will test samples directly taken from patients without the culture phase.

Before processing through PAFC system, 100 μL from each culture was removed and used for growth analysis in an H1 plate reader (Biotek, Winooski, Vermont). This procedure was done to verify the photoacoustic method and were not integral to photoacoustic testing. Growth curves were made for each culture by taking the optical density of each treated and untreated culture every minute over a 16 hour period. We determined that two hours of antibiotic treatment was sufficient to determine differential growth rates from prior experimentation. Prior to performing photoacoustic testing, treated and untreated samples

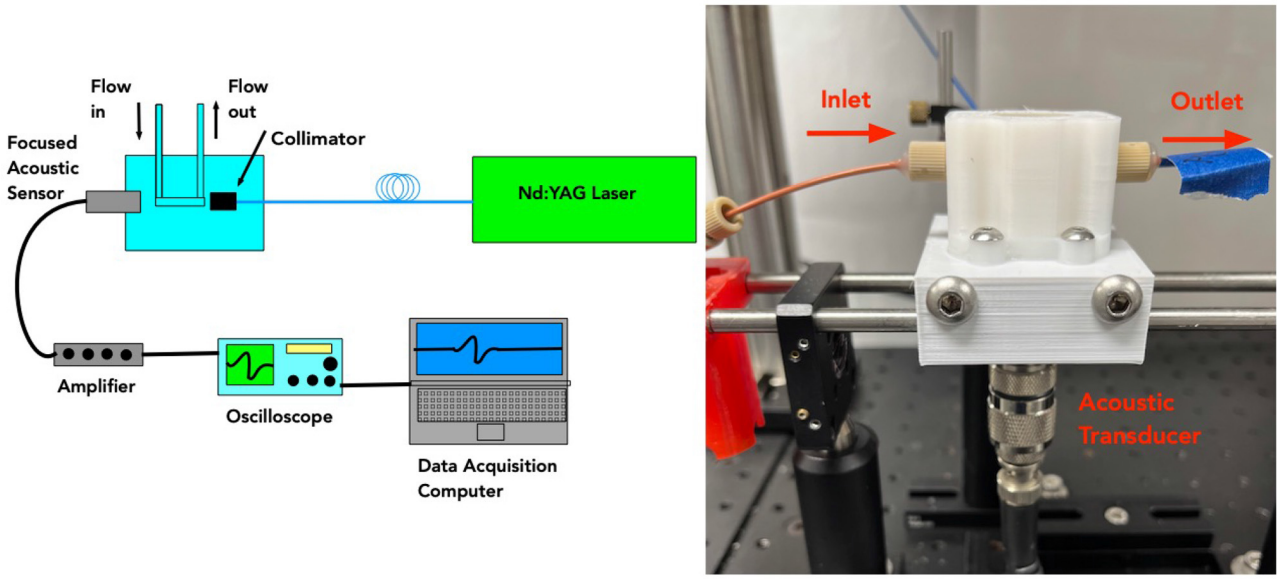

FIGURE 2
(Left) Schematic showing photoacoustic setup used for testing *S. aureus* samples. **(Right)** The actual flow chamber is shown.

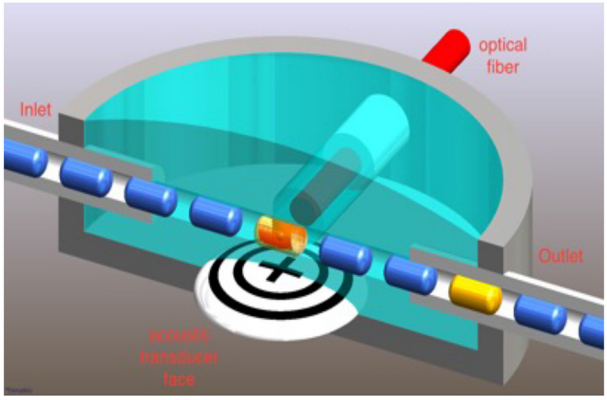

FIGURE 3
The flow chamber has alternating droplets of cell suspension and mineral oil so that detected cells are sequestered for downstream capture.

were incubated side-by-side for two hours. Photoacoustic testing of treated and untreated samples for each isolate were alternated, so that both samples were tested within twenty minutes to allow for similar growth times. Thus, total bacteria number could be compared.

2.3. Bacteriophage preparation

Bacteriophage were produced using SA113 (ATCC, Old Town Manassas, Virginia) and bacteriophage lysates were concentrated using polyethylene glycol 8000 (PEG) precipitation. Differential centrifugation and cesium chloride gradients were used to further concentrate and purify stocks to a concentration greater then 5×10^{11} plaque forming units per milliliter (PFU/mL). These bacteriophage concentration methods are previously described by Edgar et al., Nielson et al., and Yamamoto et al. (11, 26, 27). Bacteriophage were modified to increase absorbance of 532 nm laser light by adding Direct Red 81 dye (Sigma Aldrich, Saint Louis, Missouri). SP1 bacteriophage were grown using SA113. Purified phage of 10^{12} plaque forming unites per milliliter (PFU/ml) were added to a saturated solution of Direct Red 81 dye. Virion particles were then pelleted and resuspended in buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 68 mM NaCl). This process was repeated to ensure the removal of unbound dye. The absorbance spectrum of dyed phage was determined using the H1 plate reader and compared to that of undyed phage particles. Dyed phage were titred to ensure no detrimental effects were observed from the dying process. Dyed phage were retested for their ability to infect after 150 days and no difference in titer was observed.

Efficacy of Direct Red 81 to induce photoacoustic responses in bacteriophage has been previously reported (11). We also showed that bacterial cells and bacteriophage without Direct Red 81 dye showed no photoacoustic response, even at high titer. This result is consistent with the absence of optical absorption at 532 nm, the laser excitation wavelength.

2.4. k-means clustering

In order to interpret the photoacoustic data provided by the flow cytometer, we used k-means clustering to guide our differentiation between methicillin resistant and susceptible samples (28). Although a simple interpretation of the number of bacteria after oxacillin treatment compared to the number in the untreated subsample should obviously indicate whether

the bacteria was methicillin resistant or not, we used a formal method to automatically determine resistance. K-means, like other clustering methods, takes data points in a space of one or more dimensions and determines natural groupings of those points by proximity. Given a number of clusters, k, the algorithm separates all points into that number of groups. Since we are only interested in resistant and susceptible groups, we chose k = 2. We took the ratio of treated detection numbers over untreated numbers resulting in 13 numbers, approximately in the range of 0 to 1. Lower numbers would indicate that oxacillin was effective in decreasing the *S. aureus* population. However, there was no *ad hoc* threshold for determining antibiotic resistance. We applied k-means with two clusters to this data set. We used the Matlab function, kmeans, which uses Lloyd's algorithm. For simplicity, we used Euclidean distance for measuring and establishing iterative clusters. This analysis resulted in two clearly defined clusters for MRSA and methicillin susceptible samples.

3. Results

Table 1 lists each of the clinical isolates obtained from the patients. We indicate how many photoacoustic events and, hence, how many bacterial cells were detected in the oxacillin treated and untreated subsamples. Photoacoustic testing resulted in bacterial counts ranging from 2 to 689 at 2 h when incubated with oxacillin compared to 88 to 818 when samples incubated without antibiotics. Since the sample size was small and the distribution was not obviously Gaussian, we performed a nonparametric test to compare the means of the bacterial counts before and after treatment with oxacillin. Using a Wilcoxon matched pairs signed rank test, we calculated a p-value of 0.0007. Furthermore, we observed two distinct subpopulations when incubated with oxacillin. In one subgroup growth rates were similar between treated and untreated conditions, with a mean ratio of treated to untreated of 0.87, while the second group was markedly different with a mean ratio of 0.10. Once again, due to the limited sample size, we performed a nonparametric test of the two groups. Using a Mann–Whitney test, we calculated a *p*-value of 0.0012.

Seven of the 13 clinical isolates were found to be methicillin resistant using disk diffusion. Isolates found to be antibiotic resistant corresponded to the subgroup with similar growth rates. To confirm the groupings by photoacoustic detection results, we used an unbiased clustering method, k-means. The k-means column in Table 1 shows whether these numbers determined a ratio that clustered in group 1 or 2, denoting susceptible or resistant, as determined by the Matlab algorithm. The k-means algorithm resulted in 100% concordance with the known antibiotic resistance.

4. Discussion

S. aureus is a common cause of bacterial keratitis, conjunctivitis, and endophthalmitis. Given the frequency of MRSA in this population, presumptive treatment for MRSA is required until sensitivity testing can be completed. While vancomycin is often used for treatment of MRSA keratitis, it is associated with corneal toxicity (29). The clinical significance cannot be overstated, as MRSA keratitis is often part of a series of comorbidities that affect visual function. While photoacoustics can certainly identify the foundational bacterial infection and provide insight into factors that can be used to manage therapy for the infection, we are investigating ways to adapt the photoacoustic method for wider application in keratitis, which manifests in a complex environment that is still clinically challenging.

4.1. Importance of determination of antibiotic resistance

We have previously shown that this approach can be used to detect pathogens in blood and more broadly, the spread of MRSA is an increasing clinical problem for multiple types of infection in and out of hospital. Detection and treatment of MRSA have lagged behind the spread of infections (30). The emergence of resistant bacteria is compounded by prescription of non-targeted antibiotics. Rapid identification of bacterial infection is a pressing need in clinical care. It is only after identification of the pathological agent that virulence, antibiotic resistance, and other relevant factors can be considered when determining optimal antimicrobial therapy (7, 18). Misdiagnosis can result in delayed therapy which can lead to serious complications. For some infections, delayed treatment can result in sepsis, multiple organ failure, and even death. Evaluating patients suspected of bacterial infection is a complex process with unique aspects of each case that may confound proper diagnosis. A system that can immediately identify the bacterial pathogen will result in better outcomes for millions of patients each year. Over 1.5 million cases of sepsis occur annually in the United States alone (31). For patients enrolled in clinical trials, hospital mortality has fallen to about 20% (32). However, sepsis trials fail to recruit patients where the clinical diagnosis is missed and often exclude the most severely affected. Even so, mortality exceeds 30% at 90 days and 40% at one year. In the US alone, sepsis is estimated to cost more than $24 billion in hospitalization alone. Long term sequelae in survivors includes chronic lung disease, such as fibrosis, and end-stage kidney disease.

Another major reason to identify MRSA is that it requires treatment with vancomycin or other non-penicillin, non-cephalosporin, anti-staphylococcal agents. Intravenous use of these agents can produce major toxicity especially to the kidney and can result in renal failure (6). By contrast, methicillin susceptible infection can be treated with a variety of less toxic antibiotics including cephalosporins and thus avoid the risk of renal failure. However, determination of methicillin susceptibility vs MRSA can take up to 72 h using standard techniques, such as antibiotic disk diffusion and when the infection is not in the bloodstream, it sometimes cannot be determined at all (33, 34). For example, cultures are often negative with pneumonia.

Our photoacoustic system has the potential to identify and determine antibiotic resistance from patient samples. In this study, we determined antibiotic susceptibilities for 13 clinical isolates using bacteriophage tags and our photoacoustic flow cytometry system. Each isolate had previously been determined to be methicillin resistant or susceptible through disk diffusion testing.

TABLE 1 Comparison of treated and untreated photoacoustic detections of *S. aureus*.

Patient	Untreated detections	Oxacillin treated detections	k-means cluster	Disk diffusion verified by disk	Concordance: k-means and disk
K3255	798	53	1	No	Yes
K3251	726	7	1	No	Yes
B1899	611	404	2	Yes	Yes
K3282	818	689	2	Yes	Yes
K3266	119	18	1	No	Yes
K3268	88	110	2	Yes	Yes
K3270	144	134	2	Yes	Yes
K3279	198	175	2	Yes	Yes
K3261	170	135	2	Yes	Yes
K3262	183	10	1	No	Yes
K3237	137	44	1	No	Yes
K3287	210	2	1	No	Yes
K3280	227	179	2	Yes	Yes

Additionally, we made growth curves of each isolate in the presence or absence of oxacillin to reconfirm the disk diffusion classification of resistance. In each case, 16-h growth curves confirmed the disk diffusion classification as susceptible or resistant.

4.2. Cluster analysis

For each clinical isolate of *S. aureus*, one would expect the number of photoacoustic detections to be significantly fewer in the antibiotic treated sample than in the untreated sample for a methicillin susceptible strain. For a methicillin resistant strain, the detections should be roughly equal. There might even be slightly more detections in the treated sample in this case due to variability in splitting the sample and in the photoacoustic system. Simple visual inspection and ad hoc classification of results differentiated susceptible and resistant strains in this manner. To further strengthen this observation, we used k-means clustering to provide an objective means for separating the set of samples into two groups, namely, resistant and susceptible strains. K-means analysis of the photoacoustic events produced two clusters that grouped perfectly with the previously determined antibiotic resistance. Although k-means clustering was consistent with the previously determined nature of the samples, in the future, we will develop a classifier, rather than a clustering method, so that we can determine resistance or susceptibility to antibiotics from single samples in the clinic.

4.3. Accuracy of test

In this pilot study of 13 isolates, we achieved 100% concordance with disk diffusion testing for antibiotic resistance. The disk diffusion test can be considered ground truth so that classical measures of accuracy, such as sensitivity, specificity, positive predictive value, and negative predictive value, are trivially 100%, as there are no false positive or false negative results. It would be naïve to claim that the photoacoustic method has a 100% accuracy, even though this work indicates a high level of accuracy. In order to gain some measure of the usefulness of the photoacoustic method, we used a Bayesian technique to quantify the ability to determine antibiotic resistance. If we consider this experiment as a Bernoulli trial, we can use a Beta distribution as a conjugate prior which, along with the trial information, can give a posterior distribution of the probability of determining antibiotic resistance (35).

Although choice of the conjugate prior may be arbitrary, we calculated two probability distribution based on two types of conjugate priors, (1) a uniform prior, which assumes the test has an equal chance of correctly and incorrectly determining antibiotic resistance and (2) a high accuracy prior distribution with a 0.80 probability of correctly determining antibiotic resistance. For the two Beta distributions, we used Beta(α, β) = Beta(4, 4) and Beta(5, 2). The posterior distributions are shown in Figure 4 and are Beta(17, 4) and Beta(18, 1) for the uniform and high accuracy priors, respectively. The mean and variance for the uniform distribution are 0.81 and 0.007. For the high accuracy Beta, the mean and variance are 0.95 and 0.002. This brief analysis shows that even for the unlikely assumption that the test has a uniform prior probability, the accuracy is strong, with a mean probability occurring at 0.81. The more likely assumption, based on knowledge of the 13 trials and the strength of the technology, gives a mean probability of 0.95. In any case, the Bayesian analysis gives some indication of high accuracy in the absence of false positive and negatives in the data.

5. Conclusions

Rapid identification and early treatment of bacterial infections has been a goal for medicine since resistant strains emerged. Control of many bacterial strains has been put into jeopardy with the rise of antibiotic resistant strains. Early detection of bacterial

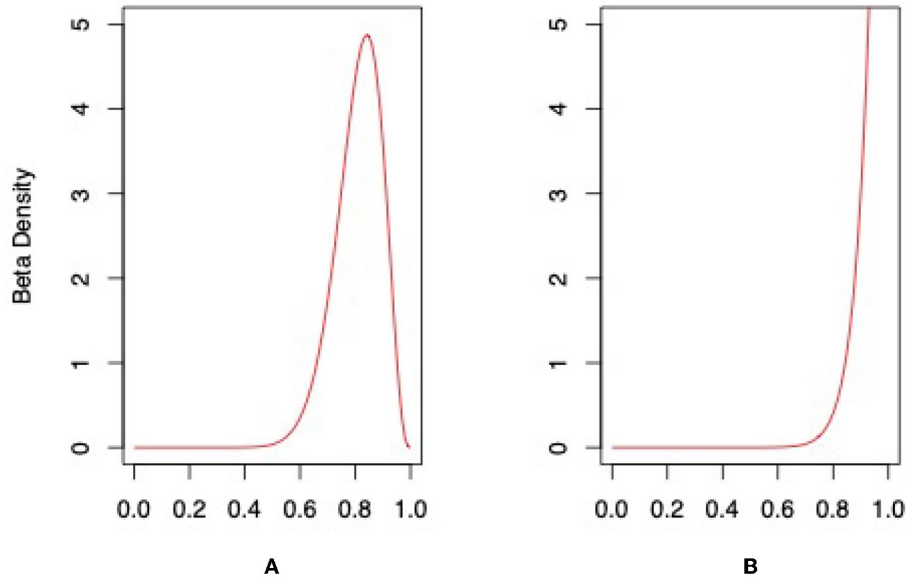

FIGURE 4
Posterior distributions for photoacoustic test using **(A)** uniform conjugate prior and **(B)** high accuracy conjugate prior.

strains and characterization of resistance is fundamental to modern clinical treatment (31). Our method exploits the specificity of naturally derived bacteriophage probes and the robust nature of laser induced ultrasonics to provide a rapid, unambiguous method for objective identification of bacterial species and their antibiotic susceptibility.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Author contributions

RE: experimental methods and data collection. A-PS, RK, JK, and JH: manuscript writing, reviewing, and technical improvements. JV: photoacoustic technology, data anaylsis, writing, and reviewing. VJ: clinical sample preparation and ophthalmalogy expertise. All authors contributed to the article and approved the submitted version.

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Conflict of interest

RE, JV, JK, and JH have equity in J3RM, LLC, a company formed to commercialize photoacoustic methods for bacterial detection, and identification. JK was employed by Spectral Medical.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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