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Original Article

Antimicrobial Susceptibility Profiles of *Staphylococcus aureus*, *Streptococcus pyogenes* and *Bacillus subtilis* Various Antibiotics

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Abstract

The rise in bacterial resistance to antibiotics necessitates the development of new antimicrobials. Pathogenic microorganisms from hospitals, communities, and environments pose significant health risks exacerbated by the uncontrolled use of antibiotics, highlighting the urgent need for innovative solutions. To address antibiotic resistance, analysed the antimicrobial activity of *Bacillus subtilis*, *Staphylococcus aureus*, and *Streptococcus pyogenes* using biochemical assays with some antibiotics: tetracycline, erythromycin, trimethoprim, rifampicin, hygromycin, and streptomycin. The study employed agar diffusion and microtiter methods to assess antimicrobial efficacy. Results indicated that all bacterial cultures were sensitive to the antibiotics tested. *Bacillus subtilis* exhibited high susceptibility to tetracycline, erythromycin, hygromycin, and streptomycin, with moderate susceptibility to trimethoprim and rifampicin. *S. aureus* showed sensitivity to the same antibiotics but to a lesser extent than *B. subtilis. S. pyogenes* were also susceptible to tetracycline, erythromycin, hygromycin, and streptomycin. Notably, the non-pathogenic *B. subtilis* demonstrated greater antibiotic susceptibility than the pathogenic *S. aureus* and *S. pyogenes*. All three bacteria exhibited consistent antibiotic susceptibility, demonstrating inhibition zones for all seven antibiotics. This study aids in developing effective treatments for resistant bacteria, contributing to combating bacterial diseases.

Keywords: Agar well diffusion, *Bacillus subtilis*, MIC, *Staphylococcus aureus*, *Streptococcus pyogenes*.

Introduction

In recent decades, antimicrobial resistance (AMR) has emerged as a critical global health issue, severely undermining the effectiveness of disease treatment and prevention within public healthcare systems (Jee *et al.*,2018). Resistant strains significantly increase healthcare costs and undermine the efficacy of first-line antibiotics, thereby heightening the risk of pervasive, difficult-to-treat infections (Ferri *et al.*, 2017). These resistant strains drive up healthcare costs and challenge the effectiveness of first-line antibiotics, escalating the hazard of widespread, intractable infections (Chaoui *et al.*, 2019). MDR bacteria are characterized by their resistance to multiple antimicrobial agents across at least three classes, presenting a formidable challenge to medical science (Chinemerem Nwobodo *et*

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al., 2022). Researchers have intensified efforts to discover novel antibiotics (Jernigan *et al.*, 2020). Nonetheless, there is a consensus that such new antibiotic classes will likely have limited durability in clinical settings (Zhang *et al.*, 2019). Employing novel compounds specifically designed to inhibit or bypass bacterial resistance mechanisms presents a promising strategy to mitigate the escalation of antimicrobial resistance (Elengoe *et al.*, 2022). Antimicrobial resistance (AMR) poses a substantial challenge to global health, resulting in increased morbidity, mortality, and healthcare costs (Shelke *et al.,* 2023). This escalating crisis over recent decades stems from the pervasive nature of highly drug-resistant microorganisms, undermining the effectiveness of treatment and preventive healthcare measures (Theuretzbacher, 2023). The emergence of (MDR) bacteria exhibiting resistance to multiple antimicrobial classes is attributable to prolonged exposure to antibacterial drugs, inappropriate antibiotic use, and inadequate infection control practices (Moo *et al.*,2020). The consequence is an increasing prevalence of bacterial strains resistant to first-line antibiotics, facilitating the transmission of these resilient strains and resulting in infections that are increasingly challenging, if not impossible, to treat (Morrison & Zembower, 2020). In response to this threat, researchers have been diligently developing novel antibiotics, although the longevity of these new drugs is often limited (Anderson *et al.*,2020). The most promising approach lies in discovering innovative compounds that inhibit or bypass resistance mechanisms, offering a glimmer of hope in the ongoing battle against AMR (Naylor *et al.*, 2018). Despite the complexity of quantifying the public health risks and financial impacts, the reality of emerging antibiotic resistance constitutes a formidable global issue (Rogers, 2021). AMR poses a severe threat to global healthcare, undermining the efficacy of both treatment and prevention strategies. MDR bacteria, fuelled by antibiotic misuse and suboptimal infection control, have increased healthcare costs and the risk of untreatable infections (Andersson *et al.*, 2020). Despite the annual introduction of new antibiotics, resistance continues to escalate due to excessive and inappropriate use. This resistance can rapidly disseminate across populations, necessitating vigilant surveillance and judicious antibiotic application (Wu *et al.*, 2022). To combat AMR, comprehensive susceptibility screening is essential prior to the deployment of new pharmaceuticals, highlighting the urgent need for innovative approaches in antibiotic development (Agha *et al.*, 2024; Fymat, 2017).

The global escalation of AMR necessitates a coordinated effort to develop new policies, invigorate research, and adopt effective management strategies (Hutchison, 2022). This study confronts the pervasive issue of MDR bacteria, which have become an omnipresent menace due to the emergence of new, resistant microbial strains. These pathogens challenge scientists to identify alternative therapeutic options, heightening the potential for additional resistance against novel medications. Accurately appraising the public health risks and the economic burden of AMR is complex; nonetheless, its threat is indisputable and recognized as a significant international concern (Velazquez-Meza *et al.*, 2022). The advent of MDR bacteria is chiefly attributed to prolonged exposure to antibiotics, misuse of such drugs, and insufficient infection control measures, leading to strains resistant to primary treatment options and increasing the risk of uncontrollable spread (Salam *et al.*, 2023). Despite the pharmaceutical industry's effort to introduce two to three new antibiotics annually, these drugs face imminent resistance due to the selective pressure exerted by widespread antibiotic use, a phenomenon well-documented in both hospital and community settings (Miethke *et al.*, 2021). Resistance determinants, especially those on mobile genetic elements, can disseminate swiftly among human and animal populations (Harris *et al.*, 2023). The variations in resistance patterns necessitate localized and regional surveillance to inform public health strategies and guide appropriate antibiotic prescription practices (Mittal *et al.*, 2023). Addressing this crisis involves screening microbial populations for susceptibility to existing antibiotics to inform the development of new treatments. While potent, synthetic alternatives carry risks and side effects that cannot be overlooked (Kadri, 2020; Norazah *et al.*, 2010). Therefore, the discovery of new drugs should proceed cautiously, considering pathogenic microorganisms' adaptive capabilities (Hamid *et al.*, 2022). The rapid rise of antibiotic-resistant bacteria globally poses a critical threat to the effectiveness of antibiotics, which have historically been lifesavers for millions (Fukunaga *et al.*, 2016). The Centers for Disease Control and Prevention (CDC) have identified several of these bacteria as urgent, serious, and concerning threats, significantly impacting both clinical outcomes and healthcare costs (Craig,

2019). There is an urgent need for well-coordinated strategies, including developing new policies and intensifying research efforts, to address this crisis (Fukunaga *et al.*, 2016; Lee *et al.*, 2018). This study focuses on the growing challenge of multi-drug resistant bacteria, which are evolving due to new mutant strains resistant to various antibiotics (Lee *et al.*, 2018). This evolution complicates the search for new, effective treatments and heightens the risk of further resistance developing against new drugs (Chinemerem Nwobodo *et al.*, 2022). This study focused on testing seven distinct antibiotics—ampicillin, tetracycline, erythromycin, trimethoprim, rifampicin, hygromycin, and streptomycin—against three bacterial species: Bacillus subtilis, Staphylococcus aureus, and Streptococcus pyogenes. The primary aim was to determine the antimicrobial susceptibility of these bacterial strains to the selected antibiotics. To achieve this, we utilized the agar well diffusion method and the minimum inhibitory concentration (MIC) technique, commonly called the microtiter method. This approach effectively assessed the antibiotics' inhibitory capacities against the targeted bacterial species (Fymat, 2017).

Material and Methods

Antibacterial assay

The effect of various antibiotics on three different bacterial strains was performed by the agar well diffusion method (Balouiri *et al.,* 2016). The minimum inhibitory concentrations (MIC) of the antibiotics against microorganisms were also determined by the micro-dilution method using antibiotic fractions serially diluted in sterile nutrient broth (Allaq *et al.*, 2021).

Selection of test microorganisms

The three bacterial strains used in the present study were the microorganisms obtained from culture, Medigene, Germany. The bacteria used were *Staphylococcus aureus, Staphylococcus pyrogens,* and *Bacillus subtilis.*

Selection of antibiotics

There were different antibiotics used in this study: tetracycline, streptomycin, erythromycin, hygromycin, rifampicin, and trimethoprim.

Minimum Inhibitory Concentration (MIC) Determination

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial ingredient or agent that is bacteriostatic (prevents the visible growth of bacteria) and is used to evaluate the antimicrobial efficacy of various compounds. The MIC was determined by observing the changes in the color of resazurin. The value of MIC was taken as the lowest concentration, which showed blue resazurin, thus indicating no sign of bacterial growth (Allaq *et al.*, 2021).

Data Analysis

All data were displayed as the mean–standard deviation. Comparisons between groups were performed using a one-way analysis of variance followed by Tukey's post hoc test. A $P < 0.05$ was regarded as statistically significant (Chaves *et al.,* 2020).

Results

Minimum inhibitory concentration (MIC) susceptibility test for B. subtilis, S. aureus and S. pyogenes

The bacterial culture, after being screened with agar well diffusion, was further tested to identify the concentration at which the bacteria could resist the antibiotics. This test was performed using the microtiter plate method (Balouiri *et al.*, 2016), where a concentration range of 0.39 to 50 mg/ml was used. *Bacillus subtilis,* when tested against some antibiotics, showed susceptibility to tetracycline, erythromycin, hygromycin, and streptomycin, while it showed slight susceptibility to trimethoprim and rifampicin, and tetracycline was used as the standard. When the antibiotics' susceptibility towards the

bacterium was tested, it was observed that tetracycline showed better results compared to the rest of the five antibiotics (Fig. 1), and the significance was $p<0.05$.

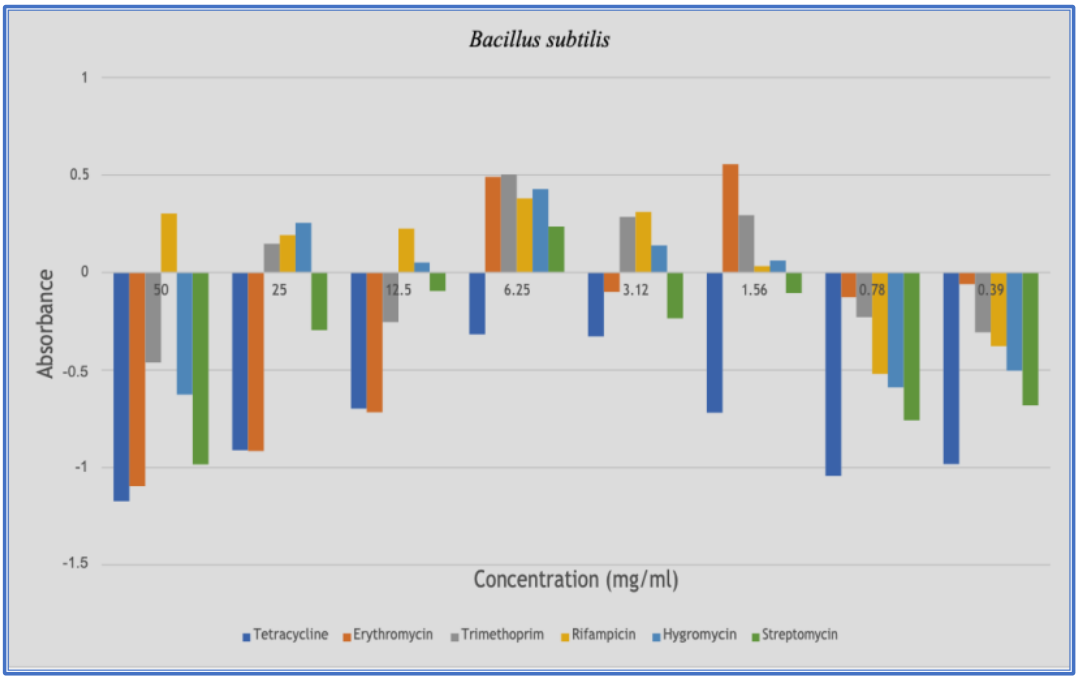

Fig. 1 Antibiotic resistance for *Bacillus subtilis* based on the MIC test

S. aureus when tested against some antibiotics, didn't show much susceptibility to tetracycline, erythromycin, hygromycin, and streptomycin, when compared to the results obtained when tested with *B. subtilis*, here, tetracycline was used as the standard. When the antibiotic's susceptibility towards the bacterium was tested, it was observed that hygromycin, rifampicin, trimethoprim, and streptomycin showed a similar trend (Fig. 2), and the significance was $p<0.05$.

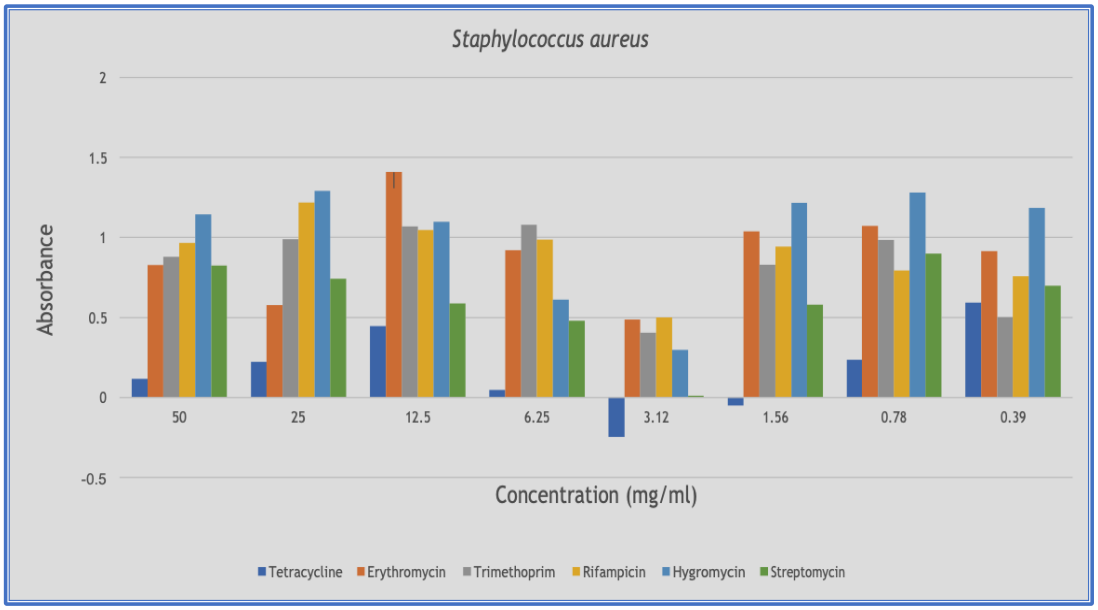

Fig.2. Antibiotic resistance *Staphylococcus aureus* based on the MIC test

S. pyogenes was tested against some antibiotics which showed susceptibility to tetracycline, erythromycin, hygromycin and streptomycin. When the antibiotics susceptibility towards the bacterium was tested, it was observed that hygromycin- erythromycin and trimethoprim- rifampicin had a similar trend (Fig.3).

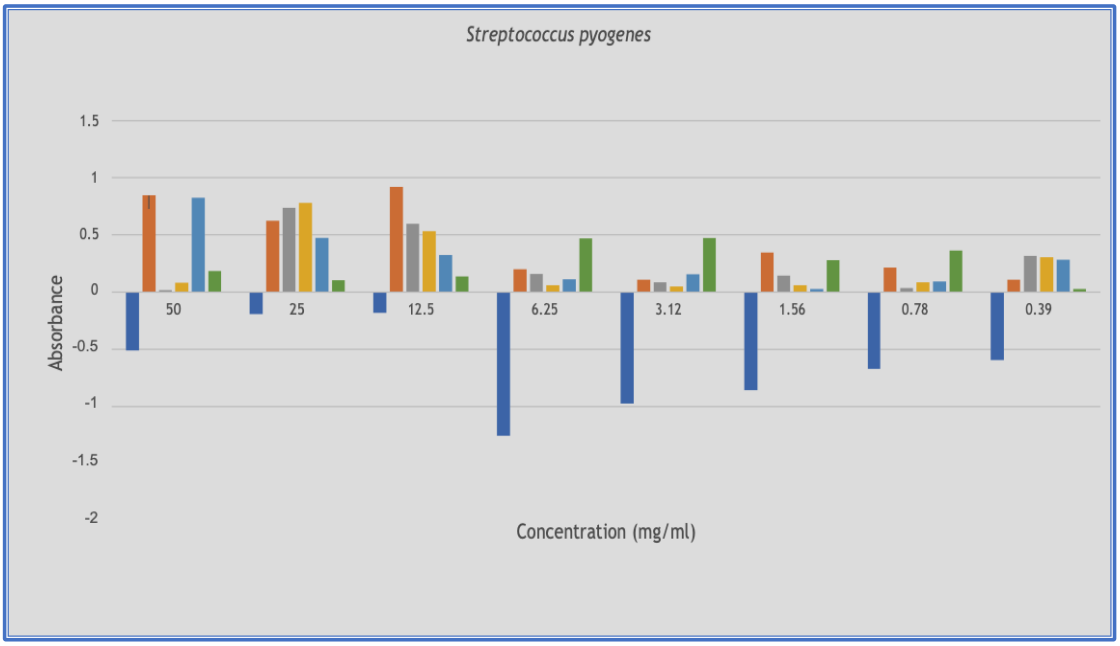

Fig.3. Antibiotic resistance *Streptococcus pyogenes*, based on the MIC test

After performing the agar-well diffusion method, it was observed that the bacterial cultures were sensitive to all the tested antibiotics. Thus, for further confirmation, the bacterial cultures were tested for their minimum inhibitor concentration using a microtiter method. This method is used because, in many cases, the agar-well method may not give a substantial result, which can lead to significant deviations in the predicted behaviour and inaccurate assessments of bacterial susceptibility to antibiotics. Thus, this MIC method is further performed on some antibiotics, wherein tetracycline was taken as a control.

In this method, *B. subtilis* was tested against some antibiotics at a concentration of 50 – 0.39 (mg/ml), wherein the bacteria showed a good result against some antibiotics except rifampicin. Since there is a lack of information on the effect of rifampicin against *Bacillus subtilis* it is difficult to compare. When the results were compared with the control, it was observed that erythromycin gave a better result compared to the rest of some antibiotics. The result obtained when the bacterium was tested against erythromycin, tetramycin, and streptomycin showed a quite similar result of sensitivity to the previously reported paper (Adimpong *et al.,* 2012; Ehling-Schulz *et al.,* 2019).

S. aureus when tested using the microtiter method against antibiotics rifampicin and hygromycin didn't show a significant result compared to the rest of the antibiotics. This result was compared with the published paper, wherein, an increasing antimicrobial effect of streptomycin, penicillin, and tetracycline (0, 10, 25, 50, 75, 150, 225, and 300 µM) on the resistant strains of *S. aureus* was observed using a method of growth curves (Camara *et al.,* 2013; Chudobova *et al.,* 2014). It showed nearly similar results to the current study.

*S. pyogene*was tested to understand the minimum inhibitor concentration of the bacterium against some antibiotics. The result for MIC is similar to that of Camara *et al.,* (2013) for erythromycin and tetracycline. When the bacterium was tested against streptomycin, and rifampicin, it showed a clear zone around the well. This result is similar to the Cattoir, (2016) and Vidya, (2017) published work, wherein the sensitivity for streptomycin was ≤0.25-0.5 µg/ml) and rifampicin was 0.12 µg/ml).

When all three studied bacteria were compared among themselves to understand the antibiotic sensitivity among them, it was found that *B. subtilis* is more sensitive towards the selected antibiotics compared to *S. aureus* and *S. pyogene* (*Bacillus subtilis >streptococcus pyogene>Staphylococcus aureus*). This might be because the other two bacteria are more pathogenic compared to *B. subtilis* (Foster, 2017; Goyal *et al.,* 2013; Ramachandran *et al.,* 2014).

Discussion

Gram-positive bacteria, characterized by their ability to retain a dark blue or violet coloration in Gram staining, are distinguishable from Gram-negative bacteria, which are unable to maintain the crystal violet stain and instead absorb the counterstain, resulting in a red or pink appearance. Gram-positive bacteria's retention of crystal violet is attributed to the high peptidoglycan content in their cell walls. Unlike Gram-negative bacteria, Gram-positive bacteria generally lack an outer membrane in their cell wall structure. In this study, we will evaluate the efficacy of commercially available antibiotic strains that are pathogenic and have been identified as multi-drug resistant (Paray *et al.,* 2023).

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a significant cause of hospital acquired infections worldwide, posing substantial challenges in healthcare settings (Lee *et al.,* 2018). Initially identified in European hospitals in the early 1960s, MRSA became notorious for its resistance to multiple antibiotics, including methicillin and gentamycin, by the 1970s in the U.S. and Great Britain (Shettar *et al.,* 2023). As a result, it is regarded as a critical clinical and epidemiological concern in hospitals, with 10 to 40% of *S. aureus* MRSA strains reported in European hospitals in recent studies (Hassoun *et al.,* 2017).

S. aureus is a Gram-positive, non-motile bacterium, often found in clusters resembling grapes, hence its name (Lowy, 1998; Williams *et al.,* 2023)**.** It is responsible for various infections, including skin infections, pneumonia, food poisoning, toxic shock syndrome, and bacteraemia. It demonstrates resistance to a wide range of antibiotics, such as oxacillin, amoxicillin, and methicillin,posing significant challenges in developing effective antibacterial agents (Tong *et al.,* 2015).

MRSA is a leading cause of surgical site infections (SSI), contributing to extended hospital stays and increased costs (Maudsdotter *et al.,* 2015). Beyond hospitals, community-associated MRSA (CA-MRSA) infections emerged in the 1990s, evolving from healthcare-associated MRSA (HA-MRSA) (Choo, 2017). This strain has since spread globally, particularly the USA300 strain in the United States. The rise of nosocomial infections due to CA-MRSA compared to HA-MRSA has heightened the urgency among researchers to control this pathogen, especially given its genetic diversity, global distribution, and other contributing factors (Miethke *et al.,* 2021; Tong *et al.,* 2015).

B. subtilis, a Gram-positive, rod-shaped bacterium, is commonly found in soil and is known as hay bacillus, grass bacillus, or Bacillus globigerina (Nagoba & Pichare, 2020).

Its ability to form endospores allows it to survive extreme temperatures and dry conditions, making it resistant even to high cooking temperatures. *B. subtilis* is recognized for its probiotic properties, beneficially impacting intestinal microflora balance, and is thus widely used in treatments for intestinal disorders (Errington & Aart, 2020). It primarily functions as an aerobe but can also operate anaerobically in the presence of nitrates or glucose. Notably, *B. subtilis* is non-pathogenic and non-toxic, with a flagellum that enables faster motility (Errington & Aart, 2020).

This bacterium has extensive biotechnological applications, including as a probiotic in dietary supplements for humans and animals and in animal feed inoculants (Adimpong *et al.,* 2012). It can stimulate the immune system (Lowy, 1998; Williams *et al.,* 2023) and produce compounds inhibitory to pathogenic microorganisms (Imperial & Ibana, 2016). The complete genome of *B. subtilis*, sequenced in 1997, comprises 4,214,810 base pairs, encoding 4,100 proteins (Adimpong *et al.,* 2012; Piewngam & Otto, 2020). However, concerns exist about its role in transferring antibiotic resistance genes, mainly as Bacillus spp. is used in commercial probiotics, some of which show resistance to various antibiotics (El-Gayar, 2017).

B. subtilis is ubiquitous, predominantly existing in a dormant spore form active *B. subtilis* produces enzymes aiding in plant degradation and can be found in human skin and intestinal tracts, though rarely colonizing (Piewngam & Otto, 2020). It also produces subtilisin, a toxin that can cause allergic reactions with repeated high-concentration exposure. *B. subtilis* has been used in enzyme production and as a broad-spectrum antibiotic (Shettar *et al.,* 2023).

S. pyogenes is a common human pathogen found in 5-15% of the population, typically on the skin or respiratory tract, often without causing any disease (Vela *et al.,* 2017). It is an extracellular pathogen capable of surviving in the host by deploying various virulence defences (Hynes & Sloan, 2016). An essential survival strategy of *S. pyogenes* involves resisting the killing mechanisms of polymorphonuclear neutrophils (PMNs), a type of white blood cell (Williams *et al.,* 2023).

Many individuals carry *S. pyogenes* without developing an illness, but it can cause mild and severe infections. Common mild infections include strep throat and impetigo, particularly in children (Avire *et al.,* 2021). However, it is essential to recognize its potential for causing severe diseases (Allaq *et al.,* 2021; Mazkour *et al.,* 2019). The bacterium's pathogenicity stems from several factors, including the production of exotoxins, streptokinase, M proteins on its surface, and its hyaluronic acid capsule, which is similar to human connective tissue, allowing the bacteria to evade the host's immune detection (Shumba *et al.,* 2019).

S. pyogenes acts as an opportunistic pathogen, often part of the normal flora in the respiratory tract, but becomes problematic when the host's defences are compromised. It can cause various diseases, from mild conditions like strep throat to severe illnesses such as necrotizing fasciitis and streptococcal toxic shock syndrome, highlighting its versatility and the importance of understanding its various pathogenic mechanisms (Hasan, 2023).

Antibiotics resistance, the need for global solutions

The emergence of multi-drug resistance in bacterial human pathogens is one of the most serious challenges for health care globally (Mancuso *et al.,* 2021). Pathogens that were previously sensitive to antibiotics are becoming resistant by acquiring resistant genes or mutations in pre-existing DNA (Mancuso *et al.,* 2021). One main source of antibiotics released into the environment is the wastewater treatment industry, which can be a hotspot for resistant gene transfer between bacteria (Taneja & Sharma, 2019). The major worldwide public health issue that needs serious action is antibiotic resistance. Enterobacteriaceae antibiotic resistance genes have been found in Enterobacteriaceae isolates from sewage plants, groundwater, and rivers (Kciuk *et al.,* 2023; Shaw *et al.,* 2021; Taneja & Sharma, 2019).

Combating antibiotic resistance

Depending on their capacity to either kill, or inhibit the growth of bacteria, they can be classified as bactericidal, or bacteriostatic, respectively (Baquero & Levin, 2021). They act by inhibiting processes essential for exponential growth, namely (i) cell wall synthesis, (ii) DNA replication, (iii) RNA transcription, and (iv) protein synthesis (Helmy *et al.,* 2023; Uddin *et al.,* 2021).

The development and accessibility of biochemical tools have provided an understanding of the mechanism of action of new compounds and provided a basis for developing new derivatives through the chemical modification of existing molecules (Salvador-Reyes & Luesch, 2015). The dynamics of the discovery of new classes of antibiotics failed in the 1960s following the introduction of quinolones (Najmi *et al.,* 2022). After 40 years, a new class of antimicrobials was introduced: oxazolidinones with linezolid (Zyvox) as the lead compound (Hashemian *et al.,* 2018; Uddin *et al.*, 2021). However, clinically significant resistance to each known class of antimicrobials has emerged within a few years following their introduction into medical usage (Fig. 4) (Hashemian *et al.,* 2018; Helmy *et al.,* 2023; Leach *et al.,* 2011).

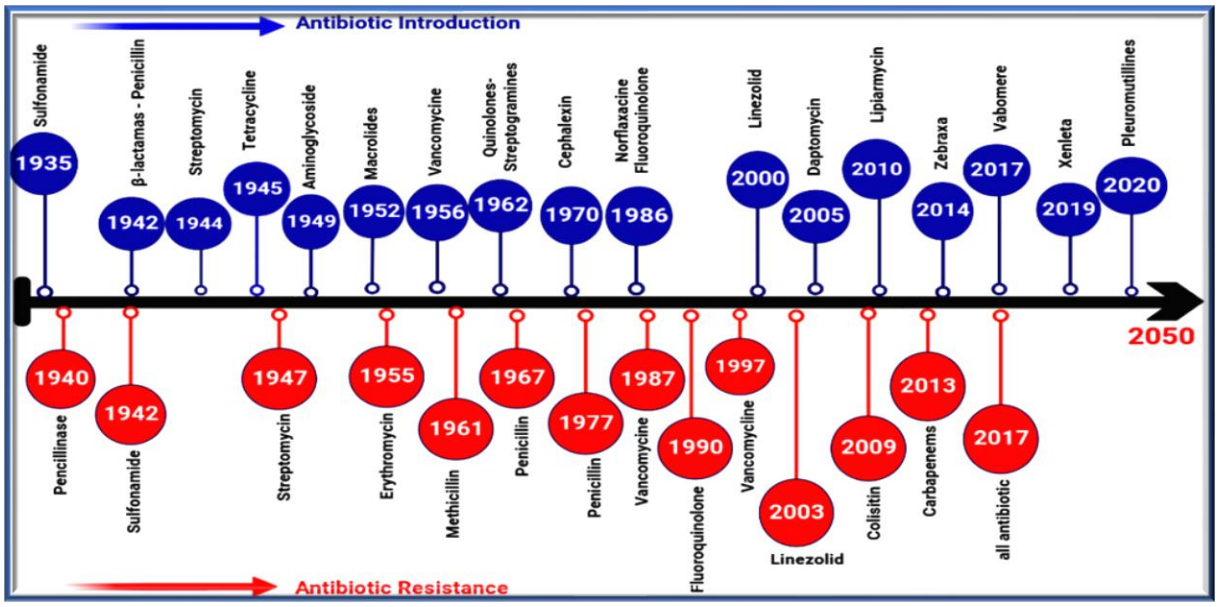

Fig. 4. The timeline illustrates antibiotics evolution. Foodborne Pathogens (Helmy *et al.,* 2023).

Conclusion

In the current study, different antibiotics were tested against three bacterial species. Antibiotics in general have been considered one of the most wonderful discoveries of mankind in the 20th century. Even though antibiotics were discovered and produced on a large scale, the need for the discovery and synthesis of new and more effective antibiotics became in high demand. The rise in antibiotic resistance among pathogenic strains from hospitals, communities, and environments has prompted increased bacterial sensitivity testing, particularly of known species. This heightened focus aims to address the urgent need for understanding and countering resistance mechanisms. Since it was found, due to the evolutionary effect, bacteria have developed resistance to already established antibiotics. Thus, the concept of studying the antimicrobial activity of known bacterial species has become essential, which will give researchers a better understanding of bacterial evolution through time from the time of its discovery.

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Conflict of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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