Comparative Evaluation of Ciprofloxacin and Azithromycin Against Biofilm Forming *Escherichia coli*

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ABSTRACT

Background and Objective: *Escherichia coli* biofilm-associated infections pose a major therapeutic challenge due to the protective nature of the Extracellular Polymeric Substance (EPS) matrix, which enhances bacterial resistance to antibiotics and host immune responses. These biofilms are responsible for recurrent urinary tract infections (UTIs) and infections associated with indwelling medical devices. The present study aimed to compare the antimicrobial efficacy of ciprofloxacin and azithromycin against *E. coli* biofilms and to explore potential synergistic effects between the two antibiotics.

Materials and Methods: Clinical isolates of *E. coli* were obtained and tested for biofilm formation using standard culture techniques. The antimicrobial susceptibility of the isolates was evaluated using the Kirby-Bauer disc diffusion method. Ciprofloxacin and azithromycin, representing fluoroquinolone and macrolide antibiotic classes respectively, were tested individually and in combination to assess their inhibitory effects. Zones of inhibition were measured to determine antibiotic efficacy and potential synergism between the two agents.

Results: Ciprofloxacin exhibited significant antibacterial activity against planktonic *E. coli* but showed reduced efficacy against biofilm-embedded bacteria, likely due to restricted antibiotic penetration and low bacterial metabolic activity within the biofilm matrix. Azithromycin demonstrated moderate inhibition of *E. coli* biofilms, attributed to its interference with quorum sensing and disruption of biofilm integrity. When used in combination, ciprofloxacin and azithromycin showed enhanced antibacterial activity, reflected by increased zones of inhibition, indicating a synergistic interaction.

Conclusion: The findings highlight the limited effectiveness of ciprofloxacin alone against *E. coli* biofilms and emphasize the potential of azithromycin to enhance its efficacy through combined action. This study supports a multifaceted therapeutic approach for managing biofilm-associated infections, incorporating combination antibiotic therapies and biofilm-targeting strategies. Further research into antibiofilm agents and molecular mechanisms of resistance is essential for developing effective interventions against persistent *E. coli* infections and mitigating the growing threat of antimicrobial resistance.

INTRODUCTION

Escherichia coli is a highly versatile, Gram-negative, rod-shaped bacterium that primarily inhabits the intestines of humans and animals, where it contributes to digestion and maintains gut health. Although, most strains are harmless, several pathogenic variants can cause serious diseases in humans and animals[1]. Pathogenic *E. coli* strains are broadly classified based on their infection mechanisms and virulence factors into enterohemorrhagic (EHEC), enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroinvasive (EIEC) and enteroaggregative (EAEC) types. Among these, EHEC strains, particularly *E. coli* O157:H7, are most notorious for causing severe foodborne outbreaks that result in hemorrhagic colitis and hemolytic uremic syndrome[2].

Infections caused by *E. coli* commonly occur through ingestion of contaminated food or water, or by direct contact with infected individuals or animals. Clinical symptoms vary from mild diarrhea to severe abdominal pain, vomiting and bloody stools. In susceptible groups-such as children, the elderly and immunocompromised individuals - these infections can lead to life-threatening complications[3].

A major factor that contributes to the persistence and pathogenicity of *E. coli* infections is the bacterium's ability to form biofilms. Biofilms are structured microbial communities encased in a self-produced extracellular polymeric matrix composed mainly of polysaccharides, proteins and nucleic acids. This matrix enables bacteria to adhere to biotic or abiotic surfaces and provides protection from environmental stresses, antimicrobial agents and host immune defenses[4]. The formation of biofilms represents a critical virulence strategy, as bacteria within biofilms display enhanced tolerance to antibiotics and immune responses, resulting in chronic and recurrent infections that are difficult to eradicate.

Antibiotic resistance in *E. coli* has become a major public health issue. The bacterium employs multiple resistance mechanisms, including β-lactamase production, efflux pumps and target site modifications, which together enhance bacterial survival under antibiotic pressure[5]. The rapid emergence of multidrug-resistant *E. coli* strains, often associated with hospital-acquired infections such as Urinary Tract Infections (UTIs), septicemia and neonatal meningitis, further complicates treatment[6].

While *E. coli* serves as a valuable model organism in genetics and biotechnology due to its simple genome and rapid growth, the pathogenic potential of certain strains necessitates ongoing efforts to develop new antimicrobial strategies and effective infection control measures[7]. Understanding the mechanisms of biofilm formation and antibiotic resistance is therefore essential for designing novel therapeutic interventions.

Biofilms are organized microbial communities that adhere to surfaces and are encased in an extracellular matrix providing mechanical stability and resistance to environmental and chemical stresses. The biofilm life cycle involves several regulated stages-initial adhesion, Extracellular Polymeric Substance (EPS) formation, maturation and eventual dispersal[8]. Biofilm-associated infections typically lead to persistent inflammation and tissue damage, as the biofilm structure protects bacteria from phagocytosis and antimicrobial penetration[9].

According to the National Institutes of Health, approximately 80% of microbial infections in humans are biofilm-associated, including those caused by *E. coli*. These infections exhibit significantly increased tolerance to antibiotics, rendering conventional antimicrobial therapies largely ineffective. Biofilms reduce antibiotic penetration, promote slow bacterial growth and facilitate horizontal gene transfer-factors that collectively enhance resistance[10].

Given this challenge, current research focuses on identifying agents that can disrupt or inhibit biofilm formation. Such biofilm-modifying substances include enzymes, metal nanoparticles, chitosan derivatives, organic acids, sodium salts, plant extracts and antimicrobial peptides. These compounds target biofilm formation at various stages or weaken the biofilm matrix, thereby enhancing antibiotic efficacy[11].

Among conventional antibiotics, macrolides and fluoroquinolones are widely used for treating *E. coli* infections. The macrolide antibiotic azithromycin exhibits multiple therapeutic effects, including inhibition of quorum sensing, suppression of biofilm formation and inhibition of bacterial protein synthesis. Its pharmacokinetic profile is favorable due to high cellular accumulation, especially within phagocytes, enabling efficient delivery to infection sites and wide tissue distribution. Azithromycin is used to treat respiratory, urogenital and skin infections, as well as chronic inflammatory conditions such as cystic fibrosis and chronic obstructive pulmonary disease[12].

Beyond its antibacterial properties, azithromycin also exerts immunomodulatory effects. It interacts with signaling molecules such as Erk1/2, NFκB and AP-1, modulating cytokine production, mucin secretion and macrophage activation. These pathways contribute to both the antibiotic and anti-inflammatory effects of the drug[13].

Ciprofloxacin, a fluoroquinolone antibiotic, is another widely used agent against *E. coli* and other Gram-negative bacteria. It acts by inhibiting DNA gyrase and topoisomerase IV-enzymes essential for DNA replication and cell division-thereby preventing bacterial growth and survival. While ciprofloxacin is highly effective against planktonic bacteria, its performance against biofilm-embedded bacteria is significantly reduced due to the protective biofilm matrix and metabolic dormancy of bacterial cells[14]. The persistence of biofilm-associated bacteria often leads to treatment failures and recurrence of infection.

Over time, *E. coli* and other pathogens such as *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Salmonella typhi* have developed resistance to ciprofloxacin, necessitating the search for combination therapies or novel antimicrobials. Co-administration of ciprofloxacin with other agents-such as bacteriophages, nanoparticles, natural compounds, or other antibiotics-has shown promise in overcoming biofilm-related resistance[15].

Biofilm-embedded *E. coli* cells can exhibit up to a 1000-fold increase in antibiotic resistance compared to their planktonic counterparts[11]. Mechanisms underlying this high tolerance include limited antimicrobial penetration, reduced metabolic activity, stress response activation and horizontal gene transfer within the biofilm microenvironment. These features collectively enable the bacteria to survive even high concentrations of antibiotics, posing a serious therapeutic challenge.

Given these complexities, understanding how antibiotics interact with biofilm-forming *E. coli* is vital. Comparative analyses of antibiotics with different mechanisms of action, such as ciprofloxacin and azithromycin, can provide insights into improving treatment strategies against chronic biofilm-associated infections. Ciprofloxacin targets bacterial DNA replication, whereas azithromycin affects protein synthesis and quorum sensing, suggesting that their combined or comparative effects could influence biofilm disruption and bacterial viability differently.

Therefore, the current study aims to evaluate and compare the efficacy of ciprofloxacin and azithromycin against biofilm-forming *E. coli*. By isolating and identifying biofilm-producing *E. coli* strains and testing their response to these antibiotics, this research seeks to contribute to the understanding of biofilm-related antibiotic resistance and to identify potential strategies for improving the treatment of persistent bacterial infections.

MATERIALS AND METHODS

Study design and location: This study was conducted in accordance with the guidelines of the Institutional Biosafety and Bioethics Committee (IBC) of the University of Agriculture, Faisalabad, Pakistan. All laboratory procedures were performed at the Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad.

Sample collection and duration: A total of 30 clinical samples were collected, including 15 urine and 15 wound surface swab samples. Urine samples were collected aseptically using sterilized containers, while wound swabs were obtained using sterile cotton swabs after cleaning the wound area with 70% ethanol to prevent contamination. Samples were collected from Allied Hospital, Faisalabad, between January and May 2024. Each sample was appropriately labeled with date and identification number, transported to the One Health Laboratory under chilled conditions (4°C) and stored until further processing.

Isolation of *E. coli*: For bacterial isolation, MacConkey agar was used as the selective medium. All media were sterilized in an autoclave at 121°C for 15 min at 15 psi. Sterile glassware, including Petri dishes and test tubes, were prepared using a hot air oven at 160-170°C for 40-60 min. Collected samples were streaked directly on MacConkey agar plates and incubated at 37°C for 24 hrs. *E. coli* colonies were identified based on typical pink coloration due to lactose fermentation.

Purification and confirmation of *E. coli*: Suspected *E. coli* colonies (pink, smooth, round) were sub-cultured on MacConkey agar supplemented with amoxicillin to obtain pure isolates. Pure cultures were incubated at 37°C for 24 hrs and subjected to morphological, microscopic and biochemical identification tests.

Colony morphology and microscopy: Colonies were evaluated for shape, size and color. Gram staining was performed to differentiate between Gram-positive and Gram-negative bacteria. The procedure involved sequential staining with crystal violet, Gram's iodine, ethanol decolorization and counterstaining with safranin. Microscopic observation at 1000× magnification revealed *E. coli* as Gram-negative, rod-shaped, motile, non-capsulated and facultative anaerobic bacteria.

Biochemical characterization: To confirm *E. coli*, standard biochemical tests were performed including Indole, Methyl Red (MR), Voges-Proskauer (VP), Citrate utilization and Catalase tests.

Indole test: The indole test determines the organism's ability to degrade tryptophan into indole via the enzyme tryptophanase. Cultures were grown in peptone water for 24-48 hrs at 37°C, followed by the addition of Kovac's reagent. A red ring at the interface indicated a positive reaction, confirming indole production.

Methyl red test: This test assesses the ability of bacteria to perform mixed acid fermentation of glucose. After incubation in glucose phosphate broth at 37°C for 48 hrs, methyl red indicator was added. A red color indicated a positive result (acidic pH<4.5), while yellow indicated negative results.

Voges-proskauer test: The VP test detects the production of acetoin, a neutral end product of glucose fermentation. Following incubation in glucose phosphate broth for 24 hrs, Barritt's reagents (α-naphthol and KOH) were added. The development of a pink-red color indicated a positive result, confirming acetoin formation.

Citrate utilization test: This test determines the organism's ability to utilize citrate as the sole carbon source. *E. coli* isolates were inoculated on Simmon's citrate agar slants and incubated at 37°C for 24-48 hrs. A color change from green to blue indicated citrate utilization and an alkaline pH shift.

Catalase test: The catalase test was performed to detect the presence of the enzyme catalase, which decomposes hydrogen peroxide into water and oxygen. A drop of 3% hydrogen peroxide was added to a bacterial colony on a glass slide. Immediate effervescence indicated a positive reaction, confirming catalase activity.

Antibiotic susceptibility testing (AST): Antibiotic susceptibility of confirmed *E. coli* isolates was determined by the Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA). A bacterial suspension equivalent to 0.5 McFarland turbidity standard was uniformly spread on MHA plates. Antibiotic discs of ciprofloxacin and azithromycin were placed aseptically on the agar surface. Plates were incubated at 37°C for 16-18 hrs. The zones of inhibition were measured in millimeters and interpreted according to the Clinical and Laboratory Standards Institute[16] guidelines. This method was used to assess antibiotic resistance patterns and determine the relative efficacy of ciprofloxacin and azithromycin against *E. coli* isolates.

Preparation of antibiotic stock solutions: Stock solutions of azithromycin and ciprofloxacin were prepared by dissolving accurately weighed antibiotic powder in sterile distilled water or saline to achieve a high-concentration solution. The stock solutions were stored at 4°C in airtight containers and diluted to working concentrations before use. These solutions remained stable for up to six months under refrigerated conditions.

Biofilm formation assay: Biofilm formation by *E. coli* isolates was evaluated using the test tube and 96-well microtiter plate methods, as described previously. Biofilm development involves sequential processes including bacterial adhesion, microcolony formation, maturation and dispersion. The 96-well microtiter plate assay was preferred due to its reproducibility and quantitative assessment capability.

Procedure:

- Fresh *E. coli* isolates were inoculated into 5 mL of Luria-Bertani (LB) broth and incubated at 37°C for 24 hrs
- A 100 µL aliquot of each culture was transferred to individual wells of a sterile 96-well flat-bottom microtiter plate
- Plates were incubated at 37°C for 48 hrs to allow biofilm formation
- Wells were gently washed twice with sterile distilled water to remove planktonic cells
- Biofilms were stained with 0.1% crystal violet for 15 min and then washed to remove excess dye
- The plates were air-dried and 200 µL of 33% acetic acid was added to solubilize the bound dye
- Optical Density (OD) was measured at 595 nm using a microplate spectrophotometer.

Each experiment was performed in triplicate and the mean OD values were calculated. The biofilm-forming capacity of isolates was classified as strong, moderate, or weak based on OD measurements compared with negative controls. The optical density cut-off (ODc) was calculated using the formula:

$$OD_c = \text{Average OD of negative control} + (3 \times \text{Standard deviation})$$

Statistical analysis: Data were analyzed using one-way analysis of Variance (ANOVA). Student's t-test was applied to compare the relative effectiveness of ciprofloxacin and azithromycin against biofilm-forming *E. coli* isolates. Differences of $p<0.05$ were considered statistically significant.

RESULTS

A total of 30 clinical samples, including 15 urine and 15 surface swab samples, were collected aseptically from Allied Hospital, Faisalabad. Out of these, nine (30%) samples were positive for *Escherichia coli* and among these, two isolates demonstrated biofilm-forming ability. All samples were transported and processed at the Institute of Microbiology, University of Agriculture, Faisalabad.

Growth of *E. coli* was initially observed in nutrient broth, indicated by visible turbidity. On MacConkey agar, well-isolated pink colonies were obtained, confirming lactose fermentation typical of *E. coli*. Colonies were round, smooth and variable in size. Each culture plate was labeled with a unique identification code and duplicate streak plates were prepared-one for biochemical characterization and the other stored at 4°C for preservation. The streak plate method successfully yielded purified *E. coli* cultures after repeated subculturing.

Microscopic examination following Gram staining revealed Gram-negative, rod-shaped bacteria, characteristic of *E. coli*. Morphological observations were further confirmed by biochemical testing, including Indole, Methyl Red (MR), Voges-Proskauer (VP), Citrate and Catalase tests. The isolates were Indole-positive, MR-positive, VP-negative, Citrate-negative and Catalase-positive, confirming their identification as *E. coli*.

In the Indole test, development of a cherry-red ring after addition of Kovac's reagent indicated a positive reaction. The MR test produced a red color, demonstrating mixed-acid fermentation. The VP test remained negative, with no color change, indicating absence of acetoin production. In the Citrate test, no color shift from green to blue was observed, confirming inability to utilize citrate as the sole carbon source. Positive catalase activity was confirmed by rapid effervescence upon addition of hydrogen peroxide, indicating the presence of the catalase enzyme.

Biofilm-forming capacity of the isolates was evaluated using the Tissue Culture Plate (TCP) method. Optical Density (OD) values of stained biofilms were measured at 595 nm using an ELISA microplate reader. Among all

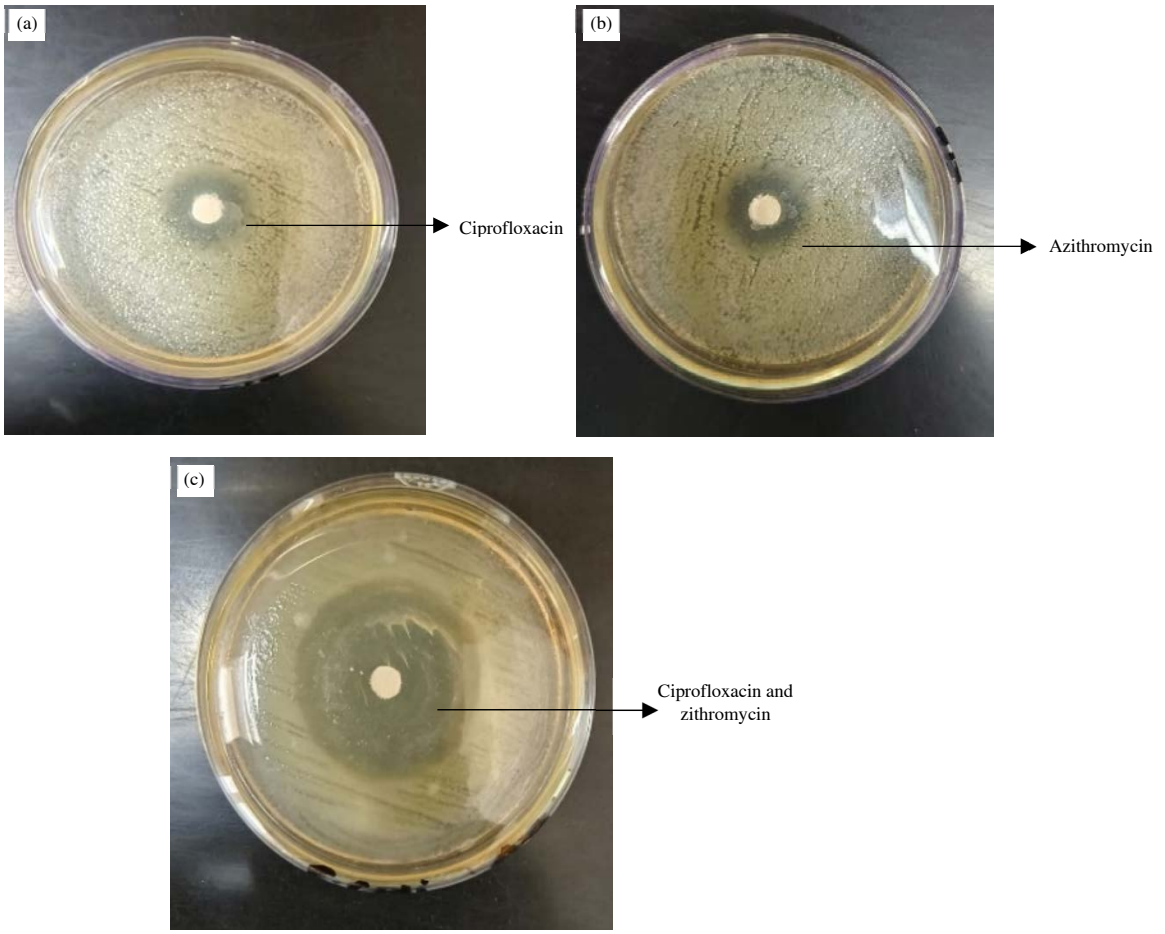

Fig. 1(a-c): Zone of Inhibition for an *E. coli* strain

Table 1: Antibiotics susceptibility testing

Bacterial strain	Ciprofloxacin zone of inhibition mm	Azithromycin zone of inhibition mm	Combine zone of inhibition mm
U1	22	No zone	23
U2	22	No zone	23
U3	19	12	21
U4	19	11	20
U5	18	No zone	20
U6	19	11	20
U7	17	No zone	18
U8	17	12	18
U9	18	No zone	19

multidrug-resistant (MDR) *E. coli* isolates, nine exhibited strong biofilm formation, six produced moderate biofilms and two showed weak biofilm production. All isolates demonstrated some degree of biofilm development, though the intensity varied among strains.

Figure 1a shows that the ciprofloxacin zone of inhibition is 19 mm for an *E. coli* strain. Figure 1b shows that the azithromycin zone of Inhibition is 12 mm for an *E. coli* strain and synergistic zone of Inhibition is 23 mm as shown in Fig. 1c.

Figure 2 illustrates the zones of inhibition produced by ciprofloxacin, azithromycin and a synergistic combination of both antibiotics. Ciprofloxacin shows a significant zone of inhibition, while azithromycin demonstrates a much smaller effect. The combination of ciprofloxacin and azithromycin results in a larger zone of inhibition, indicating a synergistic effect.

According to CLSI guidelines[16] breakpoints for Ciprofloxacin against *E. coli* are more than 21 mm for susceptible, 16-20mm for intermediate and less than 15 mm for resistant. Similarly, individual zones of Inhibition of Azithromycin against *E. coli* are normally ineffective. When these two drugs are synergistically used, an increase in zones of inhibitions is observed (Table 1).

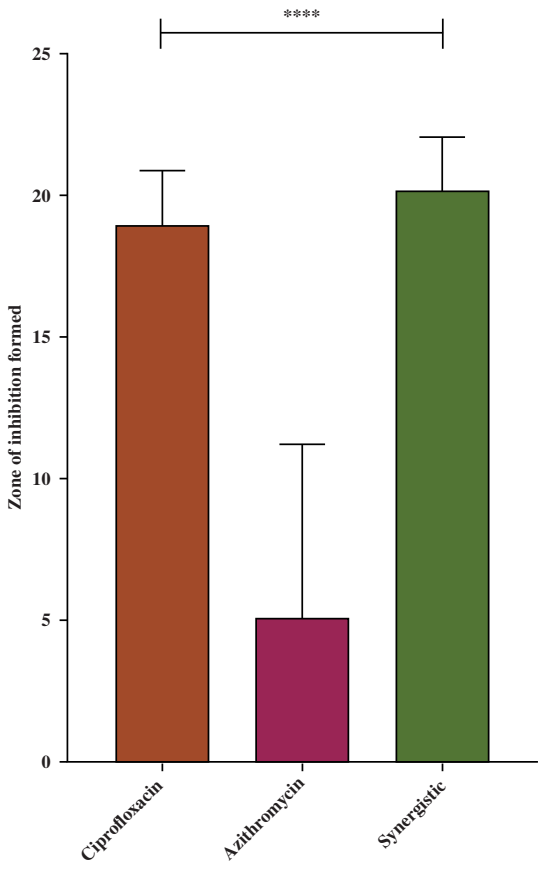

Fig. 2: Zones of inhibition created by ciprofloxacin, azithromycin and their synergistic combination

The y-axis measures the zone of inhibition in millimeters, representing the effectiveness of each treatment in inhibiting bacterial growth. The asterisks (*) denote a statistically significant difference ($p<0.0001$) between the treatments

DISCUSSION

In this study, a total of 30 samples, including 15 urine and 15 surface swab samples, were collected for the isolation of *Escherichia coli*. Out of these, nine samples tested positive for *E. coli* and two isolates demonstrated the ability to form biofilms. The antibacterial efficacy of azithromycin and ciprofloxacin was evaluated individually and in combination against these biofilm-forming isolates using the Kirby-Bauer disc diffusion method. The results revealed that ciprofloxacin exhibited greater inhibitory activity against *E. coli* compared to azithromycin, which showed notable resistance. However, the combined application of both antibiotics resulted in enlarged zones of inhibition, indicating a synergistic enhancement in antibacterial efficacy.

These findings align with earlier studies emphasizing the complex relationship between biofilm formation and antibiotic resistance in *E. coli*[17]. Biofilms, characterized by their Extracellular Polymeric Substance (EPS) matrix, play a central role in bacterial persistence by providing mechanical protection, limiting antibiotic penetration and impeding immune system responses. Consequently, biofilm-associated infections often exhibit high tolerance to antimicrobial agents, posing significant therapeutic challenges. The present study contributes to understanding the comparative efficacy of ciprofloxacin and azithromycin against biofilm-producing *E. coli*, offering valuable insights for optimizing treatment regimens targeting such infections.

Ciprofloxacin, a fluoroquinolone antibiotic, acts by inhibiting DNA gyrase and topoisomerase IV-enzymes essential for DNA replication and transcription. Although ciprofloxacin is broadly effective against planktonic bacterial cells, its reduced efficacy against biofilm-embedded cells can be attributed to the biofilm's dense EPS matrix, which limits drug diffusion and access to bacterial cells. Additionally, the slow metabolic rates of bacteria within biofilms diminish the drug's bactericidal action, as fluoroquinolones primarily target actively dividing cells[18].

Conversely, azithromycin, a macrolide antibiotic, functions by binding to the 50S ribosomal subunit, thereby inhibiting bacterial protein synthesis. Despite its limited efficacy as a monotherapy in this study, azithromycin's pharmacokinetic properties-including high intracellular accumulation and extensive tissue distribution-support its role in targeting intracellular and biofilm-associated bacteria. Moreover, azithromycin has been reported to interfere with quorum sensing, a bacterial communication system essential for biofilm formation and maintenance. By disrupting quorum sensing pathways, azithromycin can destabilize existing biofilms and increase bacterial susceptibility to antibiotics[19].

The combined use of ciprofloxacin and azithromycin in this study demonstrated enhanced antibacterial activity compared to either antibiotic alone. This synergistic interaction may result from the complementary mechanisms of action of the two antibiotics: ciprofloxacin targeting DNA replication and azithromycin interfering with protein synthesis and quorum sensing. Similar synergistic effects have been documented in previous studies, where dual antibiotic therapy enhanced biofilm disruption and bacterial eradication[20].

The persistence and resilience of biofilm-associated *E. coli* infections highlight the urgent need for novel therapeutic approaches that enhance antibiotic penetration and biofilm disruption. Combination therapies that incorporate conventional antibiotics with biofilm-targeting agents, such as quorum sensing inhibitors, dispersal agents, or biofilm-degrading enzymes, have shown potential in overcoming biofilm-mediated resistance[21]. The use of azithromycin in combination with ciprofloxacin in this study represents a promising example of such synergistic strategies.

Beyond antibiotic combinations, emerging research focuses on developing new antimicrobials with specific antibiofilm activity. Natural products, antimicrobial peptides, metal nanoparticles and other bioactive compounds have shown promise in inhibiting biofilm formation or promoting biofilm dispersal[22]. Integrating these agents with conventional antibiotics could lead to more effective treatment regimens for chronic and recurrent biofilm-associated infections.

Clinically, these findings underscore the importance of considering biofilm physiology when designing therapeutic strategies for persistent infections. Conventional susceptibility testing based on planktonic bacterial cultures often underestimates the resistance of biofilm-associated bacteria. Therefore, clinicians should interpret antimicrobial susceptibility results with caution and consider adjunctive or combination therapies when treating chronic infections caused by biofilm-forming pathogens[23].

Finally, continued research into the molecular mechanisms underlying biofilm formation, regulation and antibiotic tolerance remains critical. Advances in genomics, transcriptomics and proteomics are enhancing our understanding of the genetic determinants and signaling pathways involved in biofilm development. Such knowledge will be instrumental in identifying novel targets for therapeutic intervention and in developing next-generation antimicrobials that can effectively combat biofilm-associated infections[24].

CONCLUSION

The comparative evaluation of ciprofloxacin and azithromycin against *Escherichia coli* biofilms highlights the complexity of treating biofilm-associated infections and the limitations of conventional antibiotic therapies. Although ciprofloxacin demonstrated strong efficacy against planktonic *E. coli*, its reduced performance against biofilm-embedded bacteria emphasizes the protective role of the biofilm matrix. Conversely, azithromycin alone showed limited activity; however, when combined with ciprofloxacin, a notable enhancement in antibacterial efficacy was observed, reflected by increased zones of inhibition. These findings suggest that combination therapy involving ciprofloxacin and azithromycin can potentiate antibacterial activity and improve treatment outcomes against biofilm-forming *E. coli*. The study underscores the importance of adopting integrated therapeutic strategies that combine conventional antibiotics with agents capable of disrupting biofilm structure or inhibiting quorum sensing. Such multifaceted approaches are essential for overcoming the inherent resistance of biofilm-associated bacterial populations. By advancing the understanding of antibiotic interactions and biofilm biology, this research contributes to the development of more effective treatment protocols aimed at mitigating the growing challenge of antimicrobial resistance and improving clinical management of chronic and recurrent infections caused by *E. coli*.

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