Biomedical Letters ISSN 2410-955X DOI: https://doi.org/10.47262/BL/8.1.20211115

Research article
2022 | Volume 8 | issue 1 | Pages 50-54

ARTICLEINFO

Received
November 05, 2021
Revised
January 01, 2022
Accepted
February 15, 2022

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Keywords
Antimicrobial stress
Resistance
Inhibitory concentration
E. coli
P. aeruginosa

How to Cite
Khalid F, Bukhari H, Muzafar A, Shamim S, Fayyaz M, Iqbal A, Iqbal M. Elucidation of resistance acquisition between various genera of gram-negative bacteria. Biomedical Letters 2022; 8(1):50-54.

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Elucidation of resistance acquisition between various genera of gram-negative bacteria

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Abstract

The antimicrobial agents have the potential to maintain the selective pressure on bacteria. Bacteria can alter various mechanisms for survival under selective pressure. Thus, the bacteria acquire resistance through conjugation from co-existing bacteria during antimicrobial stress. The study was designed experimentally to investigate the acquired resistance on the basis of time against different genera of gram-negative bacteria. The study was conducted at the Department of Pathology, King Edward Medical University, Mayo Hospital, Lahore, Pakistan. *E coli* strain was sensitive to Ciprofloxacin and *Pseudomonas aeruginosa* strain was resistant to Ciprofloxacin. The macro broth dilution technique was used to verify the susceptibility of *E. coli* and *P. aeruginosa*. 0.5 MacFarland standard solution of *E. coli* and *P. aeruginosa* were mixed and kept at the sub-minimal inhibitory concentration (0.781 µg/ml) of Ciprofloxacin for 28 days. MIC of Ciprofloxacin was measured against *E. coli* at weekly basis. It was observed that the Ciprofloxacin MIC against *E. coli* after 7, 14, 21 and 28 days were <01 µg/ml, <01 µg/ml, <04 µg/ml and <25 µg/ml respectively. In conclusion, *E. coli* acquires resistance from *P. aeruginosa* under selective pressure of sub-minimal concentration of antibiotic Ciprofloxacin in 28 days.

Introduction

Antibiotic resistance is defined as ability of the bacterial pathogen to develop and acquire resistance to an antibiotic [1]. The resistance evolution among the bacterial pathogens is recognized as a major health risk to the human population. Antibiotic resistance pathogens have been identified in hospital and also in the community environment. Multi-drug resistance organisms adversely affect the patients [2]. The mortality rate of multidrug resistant infection are susceptible to antibiotics [3]. Approximately, 23,000 Americans died each year due to antibiotic resistant diseases, according to the Centers for Disease Control and Prevention (CDCP). Each year, almost $2.2 billion USD invested on antibiotic-resistant diseases [4]. The overuse of antibiotics is a key factor of antibiotic resistance development as microorganisms become resistant to medications administered more frequently than required [5]. The CDCP suggested that the inadequate antibiotic prescription is a reason of antimicrobial resistance development in United States of America [6]. Antibiotic-resistant infections are caused by self-medication [7]. Usually, the patients follow the prescription for bacterial infections and failing to complete the antibiotic course [8]. The bacterial resistance is divided into two categories as intrinsic and acquired [9]. The intrinsic resistance develops through the interaction of pathogens with the environment. Therefore the co-resident bacteria alter the working mechanism leads to develop the resistance [10]. Proteus, *Morganella morganii* and Serratia are intrinsically resistant to antibiotic colistin [11]. The acquired resistance refers to antibiotic's resistant development through different microorganisms.

Various processes, including chromosomal mutations, plasmid alterations, decreased the permeability of drugs, increased efflux of drugs, and target site modifications are used by the bacteria to gain resistance to antibiotics [12]. The mutations are the primary source to acquire resistance. The bacteria acquire antibiotic-resistant genes from other antibiotic-resistant bacteria through horizontal gene transfer. The bacterial plasmid serves as a platform for assembling and amplifying an array of antibiotic resistance genes [13].

Bacteria can be killed and inhibited by applying antibiotics. The antibiotic resistance has become a global problem. The pathogens having antibiotic resistance are a significant source of bioterrorism [14]. It was estimated that the world economy would lose $100 trillion as a result of an estimated 300 million premature deaths from antibiotic-resistant illnesses [2]. The infection resistant to drugs produce a higher death toll than do infections responsive to medicines [5, 7]. Horizontal gene transfer is performed through conjugation, transduction, and transformation. [15]. In conjugation, the plasmid facilitates the transmission of genetic material. Bacteriophages are utilized in the transduction process. The extracellular DNA material from dead bacteria is utilized by other bacteria during transformation [16].

In present study, the efforts were initiated through the analyses of *E. coli* strain and *Pseudomonas aeruginosa* strains against Ciprofloxacin. The current study revealed the resistance genes from *Pseudomonas aeruginosa* plasmid. The purpose of this study was to investigate the time required for resistance acquisition amongst various genera of gram-negative bacteria.

Materials and Methods

The study was conducted at the Department of Pathology, King Edward Medical University, Mayo Hospital, Lahore, Pakistan.

Sample selection and processing

Inclusion criteria

The pure single strain of *E. coli* was sensitive against Ciprofloxacin while as the pure single strain of *P. aeruginosa* was resistant against it. Ciprofloxacin was isolated in the clinical-pathological laboratory of King Edward Medical University, Lahore, Pakistan.

Exclusion Criteria

The resistant strain of *E. coli* and the sensitive strain of *P. aeruginosa* to Ciprofloxacin were excluded.

Methodology

The MIC of the antibiotic Ciprofloxacin was determined by using the Kirby Bauer method and the macro broth dilution method was used against the sensitive strain of *E. coli* and resistant strain of *P. aeruginosa*. Kirby Bauer method and the macro broth dilution method confirmed the susceptibility of *E. coli* and resistance of *P. aeruginosa* against the antibiotic agent Ciprofloxacin. 0.5 MacFarland standard solutions of *E. coli* and *P. aeruginosa* were made separately. 0.5 MacFarland standard solutions of *E. coli* and *Pseudomonas aeruginosa* was combined at

the subminimal inhibitory concentration of Ciprofloxacin against *E. coli* determined by using the formula $C_1V_1=C_2V_2$. The C_1 Is the concentration of stock antibiotic agent while the V_1 is the volume from stock antibiotic agent. The C_2 is the working concentration of the antibiotic agent and the V_2 is the final working volume. The concentration of the antibiotic agent was 200 µg/ml. The working concentration of the antibiotic agent and sub minimal inhibitory concentration was 0.781 µg/ml. The final working volume was 2 ml.
The $V_2 - V_1$ (2 ml - 0.008 ml), volume of broth needed for dilution was 1992 µl. The susceptible *E. coli* and resistant *Pseudomonas aeruginosa* were maintained together under the sub minimal inhibitory concentration of 4 weeks at 37° C temperature.

Macro broth dilution method

Antibiotic susceptibility testing (AST) was comprised, and the stock solution of the antimicrobial agents were prepared. An antimicrobial agent in a doubling dilution was performed and specimens of inoculums obtained from isolated colonies followed by insulation, incubation and interpretations.

Stock preparation of antimicrobial agents

The accessible antimicrobial powders prepared the stock of the antimicrobial agent. The quantity of the antimicrobial agent required for the AST process was determined by applying the formula:
Weight (mg) = $\frac{\text{Volume} \times \text{Concentration}}{}$

Potency

The concentration of the antimicrobial agent in the stock solution was ten times greater than the concentration to be tested.

Preparation of double dilutions of antimicrobial agent

For the measurement of AST, a sterile tube with a diameter of 13-19 mm was used. The tubes were closed with a loose caps screw. The final volume of 1 ml minimum was prepared through the double dilution method of antimicrobial agent in broth volumetrically.

Inoculum from isolated colonies

18-24 hours agar plate was selected to prepare the inoculum suspension in broth from the isolated colonies of *E. coli*. The turbidity of inoculum suspension in broth was adjusted according to the turbidity equivalent to 0.5 MacFarland standards. The resultant in a suspension contained approximately 1-2×10^{16} colony-forming units. The turbidity of the inoculum tube was compared with the McFarland standard tube. After 15 minutes of inoculum preparation; the suspension was diluted in broth.

Inoculation

Each tube holding 1 ml of antimicrobial agent in the diluent series (as well as a positive control tube containing only broth) was inoculated with 1 ml of the adjustable inoculum. For 24 hours, the infected tubes were incubated at 37°C in an incubator.

Interpretations

After 24 hours, the growth in the tubes was compared with the positive control in which an antimicrobial agent was not added. The definite turbidity and the amount of growth occurred in the positive control tube confirmed the validity of the analyses. MIC was defined as the lowest concentration at which an isolated strain of *E. coli* was fully inhibited (as proved by the absence of visible growth).

Results

The MIC results of Ciprofloxacin were observed against *E. coli* (**Table 1**). The subculture of the inoculated broth after 7, 14, 21, and 28 days were also analyzed. The minimal inhibitory concentration of Ciprofloxacin against *E. coli* sub-cultured at day 1, 7, 14 and 28 days were observed, and sensitivity was observed (**Table 2**). Interestingly, it was observed that there were considerable changes occurred after every seven days.
The MIC of ciprofloxacin against *E. coli* at day 1st was <01ug/ml. The MIC measured at days 7 and 14 was also <01ug/ml. However, the MIC measured at the day 21st was <3ug/ml. The MIC measured at day 28 was <25ug/ml. An all-encompassing MIC against *E. coli* was determined by analyzing the colonies that were isolated by sub-culturing the previously produced inoculum, held for 28 days.

Discussion

The antimicrobial agents are important therapeutic tools available in both human and veterinary medicine for the management and treatment of a wide range of

Table 1: Minimal inhibitory concentration by disk diffusion method of 1, 7, 14, 21 and 28 days.

MIC by Kirby Bauer Method				
Time	**Disk**	**Concentration**	**Growth**	**Interpretation**
1st Day	Ciprofloxacin	5ug/ml	Inhibited	Sensitive
7th Day	Ciprofloxacin	5ug/ml	Inhibited	Sensitive
14th Day	Ciprofloxacin	5ug/ml	Inhibited	Sensitive
21st day	Ciprofloxacin	5ug/ml	Intermediate inhibited	Intermediate sensitive
28th Day	Ciprofloxacin	5ug/ml	uninhibited	resistant

Table 2: MIC by macro broth dilution method of Ciprofloxacin at 1, 7, 21, 28 days.

MIC by Macro Broth Dilution Method										
Antibiotic dilution	**1:1**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**+ve control**	**-ve control**
Antibiotic Conc. (µg/ml)	100	50	25	12.6	6.25	3.125	1.562	0.781	Broth + inoculum	Only broth
Bacterial growth (Day 1)	-	-	-	-	-	-	-	-	+	-
Bacterial growth (Day 7)	-	-	-	-	-	-	-	-	+	-
Bacterial growth (Day 14)	-	-	-	-	-	-	-	-	+	-
Bacterial growth (Day 21)	-	-	-	-	-	-	+	+	+	-
Bacterial growth (Day 28)	-	-	-	+	+	+	+	+	+	-

bacterial infectious diseases. However, during the last several decades, the widespread use and misapplication of antimicrobial drugs in both human and veterinary medicine had resulted in the rise of bacteria strains that were no longer susceptible to antimicrobial treatment.

The antimicrobial resistant bacterial infections have an influence on both animal and human health transferred through food. The antimicrobial-resistant bacterial infection of the urinary tract, gut and lungs had caused the prolonged recovery. Antibiotic resistance has emerged as the most terrifying threat to health-care professionals [4]. The antimicrobial resistant can be natural and acquired through genetic mutation and horizontal gene transfer. Bacteria acquire resistance through a process of selective pressure of antibiotics. The non-resistant bacteria die off leaving only the resistant bacteria to flourish identical. The resistant bacteria showed high growth rate and share their antimicrobial resistance genes to the susceptible bacteria against the antibiotics. The study was designed to measure the time of horizontal gene transfer occurred between the different genera of gram-negative bacteria. For this purpose, two different gram-negative bacteria (*E. coli* and *P. aeruginosa*) were isolated in the clinical-pathological laboratory. *E. coli* strain was susceptible to antimicrobial agent ciprofloxacin and *P. aeruginosa* was resistant. *E. coli* and *P. aeruginosa* strains were kept together under the selective pressure of sub minimal inhibitory concentration of 0.781 µg/ml for 4 weeks. The MIC of *E. coli* initially measured by Macro broth dilution was <4 µg/ml. The *E. coli* strain was initially sensitive to ciprofloxacin and became resistant after 28 days. The horizontal gene transfer has occurred between *E. coli* and *P. aeruginosa* through conjugation. The *E. coli* strain acquired the antibiotic resistance genes from *P. aeruginosa* by conjugation through horizontal gene transfer. After 28 days, the MIC of *E. coli* was <25 µg/ml measured by the Macro broth dilution method. The MIC by Macro broth dilution method was measured after sub-culturing the inoculating broth on MacConkey agar for the single pure colony.

It has been evidenced that the bacteria pass through the intestine, the normal flora of the intestine not only share antimicrobial resistance genes among themselves but also communicate with the bacteria causing antimicrobial resistance. The antibiotics are given to the animals indiscriminately to enhance their growth. The antibiotic-resistant animals enter the food chain and the transfer of bacteria to the human colon through their undercooked meat. The antibiotics resistance bacteria interact with bacteria of the intestine and exchange their resistance genes among themselves to acquire resistance against the antibiotics. After that, a chain of antibiotic resistance bacteria develops from animal to human. The current study provides valuable information about the antibiotic resistance in hospital settings. The study reveals that a major cause of death in hospital settings is due to antibiotic-resistant bacterial infection spread through conjugation by horizontal gene transfer, causing prolonged treatment and no recovery from resistant bacterial infections. Eventually, this study provides the evidence which supports the theoretical arguments that antibiotic resistant infections are increasing day by day due to the overuse and misuse

of antibiotics. It exerts selective pressure on bacteria, resulting in horizontal gene transfer and the acquisition of resistance.

Conclusion

It is concluded that the indiscriminate use of oral and intravenous antibiotics puts selective pressure on the bacteria and the bacteria in order to survive in the selective pressure, acquire the resistance by conjugation through horizontal gene transfer. The susceptible strain of *E. coli* took 28 days to acquire resistance from resistance *P. aeruginosa* by conjugation through horizontal gene transfer through selective pressure of ciprofloxacin at a sub minimum inhibitory concentration of 0.781 µg/ml. Future research on horizontal gene transfer to acquire resistance in immunocompromised and nosocomial individuals should be conducted.

Acknowledgements

Authors are thankful to researchers from Department of Pathology, King Edward University, Lahore, Pakistan for continues support for this project.

Conflict of interest

The authors declare no conflict of interest.

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