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Antibiotic resistance of *Escherichia coli* isolates obtained from burn patients

Yanık hastalarından elde edilen Escherichia coli izolatlarının antibiyotik direnci

Iman Abdulqader Younis AL-SARHAN [1], Sedat ÇAM *[1]

[1]*Harran University, Faculty of Arts and Sciences, Department of Biology, 63100, Haliliye, Şanlıurfa*

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Abstract

Bacterial resistance to widely used antibiotics is an emerging global health issue and causes a huge problem in burn patients. Despite important developments in antimicrobial treatments, the risk of infection-associated mortality rate in burn patients is comparatively high. *Escherichia coli* is one of the most common causative agents of burn wound infections. Therefore, this study aimed to identify and characterize *E. coli* isolates from burn wounds using the VITEK 2 system and to test their antibiotic resistance to the most commonly used antibiotics with the disc diffusion method. In our study, of 147 clinical samples obtained from burn patients, 25 (%17) were detected as positive for *E. coli*. All these isolates were found to be resistant to cephalothin, cephradine, piperacillin, and rifampin antibiotics. The resistance to amoxicillin+clavulanic acid and ampicillin was 96% (± 8%), which was followed by amikacin and cefotaxime with a 92% (± 11%) resistance rate. On the other hand, imipenem (96% ± 8%), tetracycline (88% ± 13%), and gentamicin (76% ± 17%) were the antibiotics that showed the highest sensitivity against *E. coli* isolates. The multidrug-resistant bacteria are one of the main issues for clinical applications, so their characterization is vital in developing a proper treatment strategy. This study concluded that *E. coli* exists in burn wounds and might cause wound infection due to its resistance to different antibiotics.

Keywords: Antibiotic, Burn wound, Identification, VITEK 2

Öz

Yaygın olarak kullanılan antibiyotiklere karşı bakteriyel direnç, gelişmekte olan küresel bir sağlık sorunudur ve yanık hastalarında büyük bir probleme neden olmaktadır. Antimikrobiyal tedavilerdeki önemli gelişmelere rağmen, yanık hastalarında enfeksiyona bağlı ölüm oranı riski nispeten yüksektir. Escherichia coli, yanık yarası enfeksiyonlarının en yaygın etkenlerinden biridir. Bu nedenle bu çalışmada yanık yaralarından E. coli izolatlarının VITEK 2 sistemi kullanılarak tanımlanması, karakterize edilmesi ve en sık kullanılan antibiyotiklere karşı antibiyotik dirençlerinin disk difüzyon yöntemi ile test edilmesi amaçlanmıştır. Çalışmamızda yanık hastalarından alınan 147 klinik örneğin 25'inde (%17) E. coli bakterisi saptandı. Tüm bu izolatların sefalotin, sefradin, piperasilin ve rifampin antibiyotiklere dirençli olduğu bulundu. Amoksisilin+klavulanik asit ve ampisiline direnç %96 (± %8), bunu %92 (± %11) direnç oranıyla amikasin ve sefotaksim izledi. Öte yandan E. coli izolatlarına karşı en yüksek duyarlılığı gösteren antibiyotikler ise imipenem (%96 ± %8), tetrasiklin (%88 ± %13) ve gentamisin (%76 ± %17) olmuştur. Çoklu ilaca dirençli bakteriler, klinik uygulamalar için ana konulardan biridir, bu nedenle bunların karakterizasyonu, uygun bir tedavi stratejisi geliştirmede hayati önem taşır. Bu çalışma, E. coli'nin yanık yaralarında var olduğunu ve farklı antibiyotiklere direnci nedeniyle yara enfeksiyonuna neden olabileceği sonucuna varmıştır.

***Anahtar kelimeler**: Antibiyotik, Yanık yarası, Tanımlama, VITEK 2*

* Sedat ÇAM; sedatcam@harran.edu.tr

1. Introduction

Bacteria resistant to commonly used antibiotics are becoming a global health crisis for humans, food-producing animals, and the environment worldwide (Levy & Marshall, 2004). The use of inappropriate and excessive broad-spectrum antibiotics is one of the main reasons for this global crisis, and it requires urgent action to stop its spread (Levy & Marshall, 2004; Christaki et al., 2020). Antibiotic overuse can cause bacteria to resist routinely used antibiotics and produce adverse outcomes that can result in death, even in the most specific diseases. The only way to get out of this problematic situation is to determine effective antibiotics against specific pathogens, limiting the use of unneeded antibiotics.

Commensal *E. coli* spp. lives with their human hosts in a mutual benefit manner and does not cause diseases. It may, however, induce disease if the host's immune system is impaired or when the host's natural gastrointestinal barriers are breached, consequently playing a critical role in many infections (Bunduki et al., 2021). Due to its regular nature, *E. coli* is sensitive to many antibiotics currently used. However, *E. coli* spp. can accumulate resistance-associated genes through horizontal gene transfer (Poirel et al., 2018). They can transmit their genetic material via conjugation, transformation, or transduction, allowing genetic material to propagate horizontally in an existing population. Therefore, it has gained resistance to many antibiotics today, and this process is constantly in progress. Broad β-spectrum carbapenemase, 16S rRNA methylases, β-lactamases, plasmid-mediated quinolone resistance, and *E. coli* acquiring *mcr* genes by certain pathways have all been documented as some of the most challenging circumstances (Poirel et al., 2018).

Burns can be defined as skin/organic tissue damage resulting from the contact of heat, temperature, radiation, electricity, and some chemicals with the body. Despite ongoing improvements in patient care, infection-related burn death rates remain incredibly high (Lachiewicz et al., 2017). World Health Organization (WHO) reported that burn incidences and the number of deaths due to burn cases increase yearly. Burn cases have become a global public health threat because of the reporting of approximately 180,000 deaths caused by burns in 2020 (WHO, 2020). Burned areas are relatively vulnerable areas and, therefore, susceptible to infections. The organisms may generate a localized reaction at the burn injury site as well as a systemic response away from the burn injury site, depending on the severity and location of the burn (Moins-Teisserenc et al., 2021; Mulder et al., 2021).

According to Azzopardi et al. (2014), Gram-negative infections are the most prevalent in burn surgery. *E. coli* has been found to have a significant role in infection cases in burn centers (Azzopardi et al., 2014). Clinicians and researchers need to be able to identify these bacterial species in order to select which therapy to use. The risk of infection-associated mortality is high in burn patients (Lachiewicz et al., 2017); therefore, the characterization of *E. coli* in burn wounds is of utmost importance. In clinical settings, treating *E. coli* which has gained multidrug resistance, is extremely difficult (Poirel et al., 2018). Thus, the type of antibiotic that becomes resistant to *E. coli* and the rate of antibiotic resistance/susceptibility of antibiotics will be useful in treating *E. coli* infections in cases of burn injury. As a result, antibiotic overuse and misuse can be reduced by developing new therapeutic strategies. In this instance, the death rate can be reduced while the patient's quality of life can be improved; therefore, the current investigation aimed to identify and characterize *E. coli* isolates from burn patients and test for their resistance/sensitivity against the most commonly used different kinds of antibiotics.

2. Material and method

2.1. Sample collection

A total of 147 samples were obtained from the burn patients, who applied to Azadi and Duhok Burn Hospitals in Duhok between 1/03/2021 and 10/10/2021, collected aseptically using saline-moistened transport swabs and rapidly deposited in sterile containers. The samples were randomly selected from female or male burn patients aged 10 to 70 years old.

2.2. Morphological and biochemical characterization

The initial characterization of the isolates was made based on the colony shape, Gram staining, and biochemical assays (Atlas et al., 1995; MacFaddin, 2000). Samples were cultivated on MacConkey differential

agar media (Lab M, UK) (pH 7.2) and then incubated at 37°C for 24 hours. To distinguish lactose-fermenting (pink) bacteria from non-lactose-fermenting ones, the colonies with pink color and mucous texture were subcultured onto MacConkey agar (colorless) (Holt et al., 1994). After the 24-hour incubation at 37°C, the lactose-fermenting bacteria were re-detected.

2.3. VITEK 2 system for characterization of *E. coli*

VITEK 2 system uses a fluorogenic methodology for the diagnosis of bacterial isolates. This test identifies the microorganisms based on biochemical responses and nutrient use. A sufficient level of growth must be obtained throughout a pre-determined growth period of 18-70 hours to pass the test. It is a reasonably comprehensive approach since it includes 64 biochemical and 20 antimicrobial tests and has been widely used in clinical microbiology laboratories for strain characterization and antimicrobial susceptibility.

VITEK cards (Ref#418590) with 64 wells with nutrients and biochemical tests are employed for testing. To inoculate the card, a microbe solution is prepared. A pattern of positive and negative reactions emerges when the microbe interacts with the card. This pattern is akin to a library that gives the bacterium or yeast a name. *E. coli* strains are also one of the main strains which can be detectable in this system (Espinar et al., 2011).

A pure colony of the isolates was suspended in 3 mL of physiological saline solution to make a standardized inoculum. The bacterial suspension was compared to a standard turbid static solution (Turbidity measurement equipment) to ascertain the company's turbidity supplier. The final concentration within the tube must fluctuate between 0.5 mL and 0.63 mL. The tubes were placed in their appropriate racks. The rack containing tubes and cassette was loaded into the system and positioned in the field of fillers (filler), which automatically filled the cassette with bacterial suspension and signified the procedure's completion. The second field reader received it (reader). As the tube-filled rack travels away from the device, data from each sample is transferred to a computer attached to the VITEK system. Before the results were examined for bacterial detection, the taps were left at 37°C for 24 hours.

2.4. Molecular characterization

2.4.1. DNA isolation

The protocol used by Nessa et al. (2007) was employed for DNA extraction. The bacterial colonies cultivated MacConkey agar overnight was taken in a 1.5 mL tube filled with 200 µL of sterile distilled water. After vortexing for 15 seconds, the mixture was heated at 95°C for 10 minutes; the samples were then chilled directly in ice and the chilled suspension was centrifuged. 150 µL of supernatant was stored for PCR reactions. The quality and quantity of DNA were determined using a NanoDrop (Thermo Scientific, USA).

2.4.2. Polymerase chain reaction (PCR)

PCR reactions were performed using specific F and R primers 5'-AAAACGGCAAGAAAAAGCAG-3' and 5'-ACGCGTGGTTACAGTCTTGCG-3'. These primer pairs amplify the *uidA* gene (147 bp) encoding for the enzyme B-glucuronidase found in all *E. coli* spp. (Taha & Yassin, 2019). PCR mixture (25 µL) consisted of hot start premix (Genedirex, Taiwan), template DNA (30-100 ng L^{-1}), primer pairs (10 pmol each), and nuclease-free water with the desired amounts according to the protocol. The initial denaturation was at 95°C for 5 minutes, then followed by 35 cycles of 1 minute at 94°C for denaturation, 1 minute at 58°C for annealing, 1 minute at 72°C for extension, and a final cycle of 5 minutes at 72°C in a 9700 GeneAmp thermal cycler (Applied Biosystem). The amplified products were run in a 2% agarose gel prepared with 1 x Tris-acetate-EDTA buffer and visualized under UV light (Figure 1). The positive control was obtained from the Duhok Research Centre, College of Veterinary Medicine, University of Duhok, Iraq.

2.5. Antibiotic susceptibility test

Kirby-Bauer disk diffusion susceptibility test method was employed to determine the antimicrobial susceptibility of *E. coli* isolates to certain antibiotics listed in Table 1 (Hudzicki, 2009). The samples were suspended in 5 mL of sterile 0.85% sodium chloride and well-shaken. The density of the samples was adjusted based on 0.5 McFarland standards (equivalent to ~1.5 x 10^8 CFU mL^{-1}). Using a sterile cotton swab, the

bacteria from the suspension were placed on a Mueller-Hinton agar (Difco™) plate together with certain antibiotics at well-spaced points from each other. The antibiotic discs were firmly placed over the plates to ensure contact with the agar. Following 24-hour incubation at 37°C, sensitivity/resistivity was evaluated based on the presence/absence of inhibition zones around the antibiotic disk. The zone diameter was measured and evaluated according to the standard chart of the manufacturer (Ref#418590) based on the US Clinical and Laboratory Standards Institute (CLSI) guidelines.

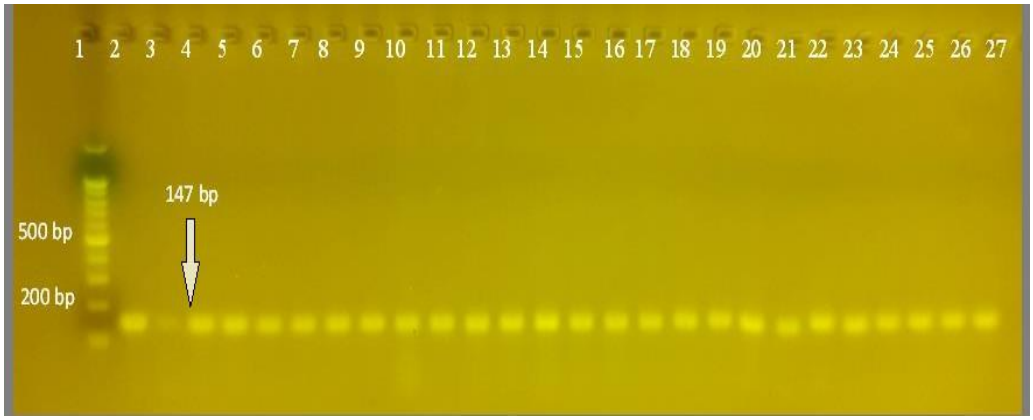

Figure 1. Confirmation of *E. coli* isolates based on *uidA* gene (147 bp). Line 1: 100 kb DNA marker (Addbio, Korea); Line 2-26 samples; Line 27: Positive control

Table 1. Antibiotics used in this study

No.	Antibiotics	Abbreviation	Disc symbol
1.	Amikacin	AK	30
2.	Amoxicillin + Clavulanic acid	AMC	20/10
3.	Ampicillin	AM	25
4.	Cefotaxime	CTX	30
5.	Ceftriaxone	CRO	30
6.	Cephalothin	KF	30
7.	Cephradine	CE	30
8.	Ciprofloxacin	CIP	5
9.	Doxycycline	DO	30
10.	Gentamicin	GM	10
11.	Imipenem	IPM	10
12.	Piperacillin	PRL	100
13.	Rifampin	RA	30
14.	Sulfamethoxazole + Trimethoprim	SXT	1.25/23.75
15.	Tetracycline	TE	30

2.6. Statistical analysis

The percent confidence interval (CI) of the samples was calculated based on the 95% confidence level by the following formula (Hazra, 2017).

$$\text{CI} = \text{Sample proportion (p)} \pm \text{z value} \times \text{Standard error of proportion}$$

$$p \pm z\,\text{value} \times \sqrt{\left[p\frac{(1-p)}{n}\right]} \quad (1)$$

3. Results and discussion

In the recent decade, Gram-negative bacteria such as *E. coli* have developed resistance against routinely used antibiotics, possibly due to antibiotic misuse. This scenario has practically reached a critical point for patients, mainly burn patients, which can result in various adverse outcomes, including death (Kakoullis et al., 2021). Infection and sepsis are considered the leading causes of death in burn cases (Vinaik et al., 2019). Bacteria can quickly infect and colonize skin wounds. According to current statistics, present antimicrobial therapies are insufficient due to bacterial resistance, necessitating the development of novel treatment approaches (Kakoullis et al., 2021). In addition, the detection of the bacterial species causing the infection and subsequent characterization of the bacteria are necessary for the development of innovative treatment techniques (Lachiewicz et al., 2017; Vinaik et al., 2019); therefore, this study characterized *E. coli* from burn patients and tested their resistance/sensitivity against commonly used antibiotics.

A total of 147 samples were taken from patients of different age groups and gender at two hospitals in Duhok. According to morphological, biochemical, and molecular analyses, of 147 samples, twenty-five (17%) were detected as positive for *E. coli*. The resistance of the *E. coli*-positive samples to 15 commonly used antibiotics was investigated using the disc diffusion method. As a result, all *E. coli* isolates were found to be resistant against cephalothin, cephradine, piperacillin, and rifampin (Table 2). 24 samples (96% ± 8%) showed resistance against amoxicillin + clavulanic acid and ampicillin, while 23 samples (92% ± 11%) were found to be resistance against amikacin and cefotaxime antibiotics (Table 2 and Figure 2). On the other hand, imipenem (96% of samples), tetracycline (88% of samples), and gentamicin (76% of samples) were the antibiotics that conferred the highest sensitivity against the isolates tested (Figure 2).

Despite being the first commercially available cephalosporin, the cephalothin antibiotic, often known as cephalothin, is still widely used (Nascimento et al., 2021). All *E. coli* samples in our investigation developed resistance, most likely as a result of its extensive use over many years. According to the study conducted by Raeispour & Ranjbar, (2018), *E. coli* exhibited great resistance to cephalothin. They found that the ratio of cephalothin resistance was 74% in 60 *E. coli* isolates. In another study, this ratio was 76.9-100% (Jung et al., 2021). Furthermore, cephradine, another antibiotic to show high resistance against *E. coli* in our study, is generally used to treat upper respiratory, skin, ear, and urinary tract bacterial infections. Although all cases (100%) showed resistance against cephradine in our study, this ratio was found to be lower (68.7%) in another study conducted on 85 patients with concurrent UTI and diabetes (Shill et al., 2010). This might be explained by the use of different strains isolated from different locations. Conducting complete and detailed studies can help to clarify the different results obtained by the studies.

Antibiotic development processes are known to develop through intrinsic, acquired, and adaptive pathways in general (Lee, 2019). However, horizontal gene transfer of enzymes destroying/modifying antibiotics such as β-lactamases including penicillinases, ESBL, AmpC enzymes, and carbapenemase across bacteria is the most common cause of microbial resistance. Resistance to first-generation cephalosporin antibiotics like cephalothin and cefradine has been found to develop via this mechanism (Bunduki et al., 2021; Jung et al., 2021). Another well-understood process for antibiotic resistance development is mutations in the 23S rRNA, topoisomerase IV, and RNA polymerase β subunit. When antibiotics are given to a bacterial culture, certain bacteria develop antibiotic resistance due to gene mutations, and those that grow resistant over time become the majority species in the community (Christaki et al., 2020; Bunduki et al., 2021).

Piperacillin, a broad-spectrum-lactam antibiotic, has been frequently used with beta-lactamase inhibitors like tazobactam. Piperacillin is classified as an antibiotic of essential importance to human medicine by WHO because it is so effective and widely used. Of 580 *E. coli* samples taken from wastewater treatment plants in South Africa, 246 (42.4%) were shown to have multidrug resistance, including piperacillin (Mbanga et al., 2021). However, all the isolates tested in our work were resistant to piperacillin. This difference might be due to bacterial collection from different sources. Unlike that study, we obtained samples from infected patients. Environmental and clinical samples may respond to the treatments differently. It is well-established that

increased environmental pollution leads to the enhanced selection of antimicrobial-resistant microorganisms because of the co- and/or cross-selection of antibiotic resistance genes (Buelow et al., 2021), which may explain the difference in antibiotic resistance between environmental and clinical isolates. The usage of piperacillin should be re-evaluated in light of our and similar findings to reconsider the areas of use/case and combinations. The resistance of *E. coli* against piperacillin-tazobactam antibiotic was attributed to the extensive amplification of the genes such as the S26-associated bla_{TEM-1} gene (Schechter et al., 2018; Hansen et al., 2019). It was suggested that *E. coli* upregulates the respective gene, increasing Bla_{TEM-1} protein levels to overcome the inactivation mechanisms of the piperacillin-tazobactam antibiotics when exposed to their subinhibitory concentrations.

Table 2. Sensitivity or resistance of *E. coli* isolates against different antibiotics

	Antibiotics	Amikacin	Amoxicillin + Clavulanic acid	Ampicillin	Cefotaxime	Ceftriaxone	Cephalothin	Cephradine	Ciprofloxacin	Doxycycline	Gentamicin	Imipenem	Piperacillin	Rifampin	Sulfamethoxazole + Trimethoprim	Tetracycline
Samples codes	A01	R	R	R	R	R	R	R	S	R	S	S	R	R	R	S
	A02	R	R	R	R	R	R	R	S	R	S	S	R	R	R	S
	A03	R	R	R	R	R	R	R	S	S	S	S	R	R	R	S
	A04	R	R	R	R	R	R	R	S	S	S	S	R	R	R	S
	A05	S	R	R	R	R	R	S	S	S	S	S	R	R	R	S
	A06	R	R	R	R	R	R	R	S	R	S	S	R	R	R	S
	A07	R	R	R	S	R	R	R	R	R	S	S	R	R	R	S
	A08	R	R	R	R	S	R	R	S	R	R	S	R	R	R	S
	A09	S	S	S	R	R	R	R	S	S	R	S	R	R	R	R
	A10	R	R	R	R	R	R	R	R	R	S	S	R	R	S	R
	A11	R	R	R	R	R	R	R	R	S	S	S	R	R	S	R
	A12	R	R	R	R	R	R	R	S	R	R	S	R	R	S	S
	A13	R	R	R	R	R	R	R	S	R	R	S	R	R	R	S
	A14	R	R	R	R	R	R	R	R	S	S	R	R	R	R	S
	A15	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S
	A16	R	R	R	R	S	R	R	R	S	S	S	R	R	R	S
	A17	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S
	A18	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S
	A19	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S
	A20	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S
	A21	R	R	R	R	R	R	R	R	S	S	S	R	R	R	S
	A22	R	R	R	R	R	R	R	R	R	S	S	R	R	R	S
	A23	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S
	A24	R	R	R	R	S	R	R	R	R	S	S	R	R	R	S
	A25	R	R	R	S	R	R	R	R	R	S	S	R	R	R	S
Total (R)		23	24	24	23	22	25	25	15	13	6	1	25	25	22	3
Total (S)		2	1	1	2	3	0	0	10	12	19	24	0	0	3	22

Rifampicin, another antibiotic in our study that had 100% resistance, was developed in 1965 and has been commercially accessible since the late 1960s (Grobbelaar et al., 2019). In bacterial cells, rifampicin inhibits RNA polymerase, halting mRNA production (Hamouche et al., 2021). However, resistance to rifampicin (RIF) is challenging due to the abundance of mutations in the β subunit of RNA polymerases resulting in decreased affinity for rifampicin (Goldstein, 2014). This may lead to misleading results. Especially since RNA polymerase β subunit mutations in *E. coli* are the deciding factors in resistance to rifampicin, it is critical to identify existing mutations and to continue research on this topic (Feklistov et al., 2008; Goldstein, 2014). Excessive usage of β-lactams antibiotics like rifampicin causes *E. coli* to develop resistance to rifampicin. Thus, the usage of agents with rifampicin is still being studied to solve this problem. In this connection, it was reported that using cationic polyurethane with rifampicin simultaneously diminishes *E. coli* resistance by up to 64 times (Tantisuwanno et al., 2021; Verma et al., 2021).

On the other hand, imipenem (96% ± 8%), tetracycline (88% ± 13%), and gentamicin (76% ± 17%) were the antibiotics that showed a high sensitivity against *E. coli* in our investigation. Similarly, in recent works, the imipenem's sensitivity was reported to be 100% (Raeispour & Ranjbar, 2018). This high sensitivity was also confirmed by relatively comprehensive meta-analysis studies conducted from 1991–2015. In this analysis, among 35, 118 cases in total, imipenem with an 86% ratio showed the highest sensitivity against *E. coli* among widely used antibiotics worldwide (Mortazavi-Tabatabaei et al., 2019).

Tetracyclines, which have a broad spectrum of antibiotic action, are commonly used to treat skin conditions like acne, as well as infections of the intestinal, respiratory, and urinary tract. *E. coli* develops resistance to tetracycline by horizontal transfer of the efflux pump-encoding genes, enzymatic degradation genes, or the genes encoding ribosome protective proteins (Berglund et al., 2020). In a study conducted with 128 newborn babies, similar to our results, tetracycline resistance was found in 12% of the samples (Karami et al., 2006). Due to showing high susceptibility against *E. coli*, as supported by our findings, it is still routinely utilized in many infectious disorders. According to Karami et al. (2006), the fact that tetracycline resistance has not decreased in *E. coli* over the last two decades may suggest that the frequent use of tetracycline in human medicine offsets the reduced use of tetracycline in livestock. Excessive use, however, may reduce *E. coli* susceptibility in the future unless controlled.

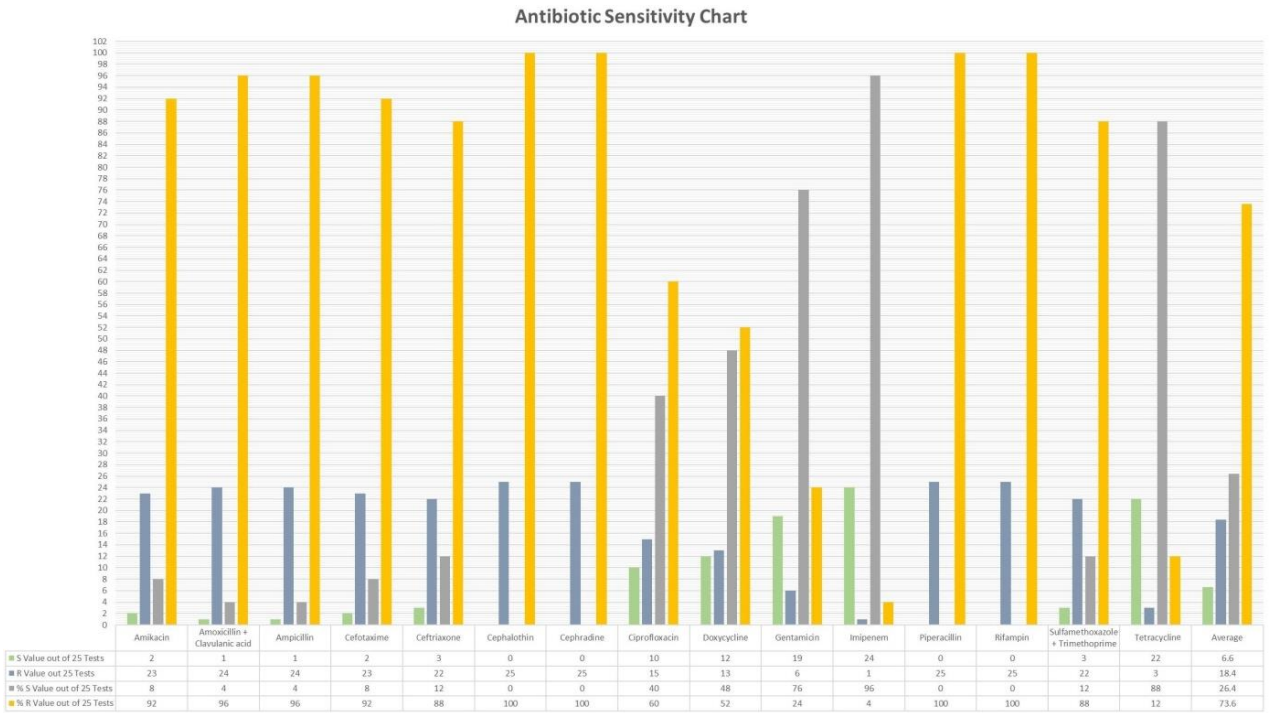

Figure 2. The ratio of the susceptibility/resistance of *E. coli* isolates against widely used antibiotics

Gentamicin has been commercially available for medical use since 1964 and is still on the WHO's List of Essential Medicines. It is commonly used to treat infections caused by a variety of bacteria, primarily *E. coli*. According to our findings, *E. coli* continues to be very susceptible to gentamicin. However, over the years, it

has been observed that this sensitivity is steadily reducing (WHO, 2019). While the rate of resistance against gentamicin was 5.36% in 2014, this rate was determined as 6.7% in 2019. This tendency indicates increasing gentamicin resistance, which in parallels with the data of WHO (Ong et al., 2021).

4. Conclusions

Bacteria resistant to routinely used antibiotics are posing a global health threat to humans. Overuse of antibiotics can result in resistance to commonly used antibiotics and harmful consequences that can lead to death. Although *E. coli* is a beneficial bacterium in its natural habitat, it has been linked to various diseases. Gram-negative infections, such as *E. coli*, are the most common in burn surgery, and their identification is critical for the development of innovative therapeutic options to prevent antibiotic usage and misuse. In our study, *E. coli*-positive samples were found to be the most resistant to cephalothin, cefradine, piperacillin, and rifampin, whereas they were the most sensitive to imipenem, tetracycline, and gentamicin. Our findings appear to be in line with those of previous long-term, comprehensive, and global investigations.

Author contribution

The authors equally contributed to the article.

Declaration of ethical code

Ethical permission was approved by Reserch Ethics Committee from Ministry of Health Duhok Directorate General of Health and Ministry of Higher Education University of Duhok with a reference number (11112020-5-3) on 11th November 2020.

Conflicts of interest

The authors declare no competing interests.

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