Istanbul J Pharm 53 (1): 45-50
DOI: 10.26650/IstanbulJPharm.2023.1156339
Original Article

Antibiotic susceptibility and biofilm formation of multi-drug resistant Gram-negative bacteria

Ozlem Oyardi[1], Mayram Hacioglu[2], Fatima Nur Yilmaz[2], Nese Inan[3], Ayse Seher Birteksoz Tan[2]

[1]Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Turkiye
[2]Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Istanbul, Turkiye
[3]Synevo Laboratories, Microbiology Laboratory, Ankara, Turkiye

ORCID IDs of the authors: O.O. 0000-0001-9992-7225; M.H. 0000-0003-0823-631X; F.N.Y. 0000-0001-8442-8538; N.I. 0000-0002-1559-6244; A.S.B.T. 0000-0001-8767-710X

Cite this article as: Oyardi, O., Hacioglu, M., Yilmaz, F.N., Inan, N., & Birteksoz Tan, A.S. (2023). Antibiotic susceptibility and biofilm formation of multi-drug resistant Gram-negative bacteria. *Istanbul Journal of Pharmacy, 53(1), 45-50.* DOI: 10.26650/IstanbuLJPharm.2023.1156339

ABSTRACT

Background and Aims: Gram-negative bacteria are important pathogens that can cause community- and hospital-acquired infections as well as opportunistic infections, with antimicrobial resistance in Gram-negative bacteria becoming a growing crisis in clinical medicine. Biofilm formation is one of the mechanisms of bacterial resistance, which makes bacteria less susceptible to antimicrobial agents and unable to be killed by host immune mechanisms. Therefore, this study investigates the antimicrobial resistances and biofilm-forming abilities of a total of 98 Gram-negative strains isolated from various clinical specimens.

Methods: A disc diffusion assay was performed to detect the susceptibility profiles of 98 Gram-negative strains. The biofilm-forming abilities of strains were also determined using the Crystal Violet assay.

Results: Concerning the disc diffusion assay, most of the isolates were found to be resistant to carbapenems, with more than 90% of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Escherichia coli* isolates being found resistant to ceftazidime and piperacillin. Most of the *Pseudomonas aeruginosa* isolates (75%) were found to be resistant to imipenem and aztreonam. All isolates had the ability to form biofilms. Overall, 56% of isolates were strong formers, and 29% were moderate biofilm formers. Strong biofilm formation was observed in most strains except for *K. pneumoniae*.

Conclusion: The surveillance of susceptibility profiles and biofilm formation is important for determining their variable susceptibility patterns and aiding in the appropriate management of infections caused by these organisms.

Keywords: Multi-drug resistance, antibiotic susceptibility, biofilm

INTRODUCTION

Gram-negative bacteria differ from Gram-positive bacteria in that they have a thinner peptidoglycan layer and an outer membrane that acts an important mechanical barrier. They are very common in nature and cause many serious infections (Eichenberger & Thaden, 2019). Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii* are some of the Gram-negative bacteria that most commonly cause nosocomial infections such as ventilator-associated pneumonia, urinary tract infections, and sepsis and that develop resistances due to the inappropriate use of antibiotics (Hammoudi & Ayoub, 2020). Antimicrobial resistance can occur through numerous mechanisms, including antibiotic degradation by enzymes, impermeability of the bacteria to the antibiotic, antibiotic target modification, genetic transfer of resistance genes, and increase in bacterial membrane permeability (Eichenberger & Thaden, 2019; Vivas, Barbosa, Dolabela, & Jain, 2019). Due to the natural structure of the outer

Address for Correspondence:
Ozlem OYARDI, e-mail: ozlemyrd@gmail.com

Submitted: 07.08.2022
Revision Requested: 21.10.2022
Last Revision Received: 05.10.2022
Accepted: 12.12.2022
Published Online: 27.03.2023

membrane, Gram-negative bacteria are naturally resistant to some large-scale antibiotics such as vancomycin. In addition, modifications in the outer membrane such as changes in hydrophobic properties and porin changes in the outer membrane may cause resistance to develop. Thus, Gram-negative bacteria tend to be more resistant to antibiotics than Gram-positives (Breijyeh, Jubeh, & Karaman, 2020).

Bacteria are defined as multi-drug resistant if they are resistant to three or more antimicrobial classes (Magiorakos et al., 2012; Perdikouri et al., 2019). Multi-drug resistance is an important factor that increases the length of hospitalization stay, mortality, and cost of treatment (Peters et al., 2019; Thaden et al. 2017).

The ESKAPE group is an important group of bacteria that cause nosocomial infections and are able to avoid the effects of antibiotics with resistance mechanisms. Gram-negative bacteria form a large part of this ESKAPE group, which gets its name from the first letter of the following bacteria: *Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumanni*, *P. aeruginosa*, and *Enterobacter* species (Rice,, 2008). These microorganisms are serious threats to hospitals because they can easily contaminate hospital surfaces and medical equipment. In 2019, The Centers for Disease Control and Prevention (CDC) reported carbapenem-resistant *Acinetobacter* spp. and carbapenem-resistant *Enterobacteriaceae* to be urgent threats requiring immediate and aggressive action due to their high risk of outbreaks in hospitals and nursing homes.

Multi-drug resistance is not the only cause of treatment failure; some bacteria also have the ability to form biofilms can make them up to 1,000 times more resistant to antibiotics. Biofilms are communities of microorganisms embedded in a self-produced exopolysaccharide matrix containing various substances such as polysaccharides, proteins, and DNA (Cepas et al., 2019; Wang, Zhao, Chao, Xie, & Wang, 2020). Biofilms can form on biotic and/or abiotic surfaces. The formation of biofilms on medical devices such as ventilators and implants applied externally to patients poses a serious risk in terms of hospital infections. Biofilm formation is a complex process consisting of the following many stages: attachment, micro-colony formation, maturation and formation of the architecture of the biofilm, and detachment (Jamal et al., 2018). Biofilm forming ability causes bacteria to become more resistant to antibiotics and bodily defense mechanisms. Gram-negative bacteria with acquired antimicrobial resistance and biofilm-forming ability are a very serious threat causing nosocomial infections. This study thus aims to evaluate the antimicrobial resistances and biofilm formations of a total of 98 Gram-negative strains.

MATERIALS AND METHODS

Strains

A total of 98 Gram-negative bacteria, including 37 *Acinetobacter baumannii*, 34 *Klebsiella pneumoniae*, 16 *Pseudomonas aeruginosa*, 5 *Escherichia coli*, 4 *Stenotrophomonas maltophilia*, 1 *Serratia sp.*, and 1 *Enterobacter sp.* isolates from various specimens including blood, urine, sputum, abscess, tracheal aspirate, and bronchoalveolar lavage fluid were obtained from the Synevo Laboratories Ankara Central Laboratory in Turkey (2020-2021). Identification of the strains was performed using the Vitek 2 (BioMerieux, France), API 20 E, and API 20 NE (BioMerieux, France) systems. Before the analyses, each isolate was cultured on tryptic soy agar (TSA, Difco Sparks, MD, USA) plates to ensure viability.

Disc diffusion assay

Imipenem (10 µg), meropenem (10 µg), gentamicin (10 µg), tobramycin (10 µg), amikacin (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ceftazidime (30 µg), cefepime (30 µg), piperacillin (100 µg), and aztreonam (30 µg) discs (Bioanalyse, Turkey) were used for the antibiotic susceptibility testing. The antimicrobial susceptibility assay was performed using the disc diffusion method on Mueller-Hinton agar (MHA-Sigma-Aldrich, St. Louis, MO, USA) in accordance with the Clinical and Laboratory Standards Institute (CLSI) M100-Ed 31 (CLSI, 2021). Bacterial suspensions were prepared by selecting similar colonies from an overnight culture with a sterile loop and suspending the colonies in sterile saline (0.85% NaCl w/v in water) at the density of a McFarland 0.5 standard, approximately corresponding to 1–2 x 10^8 cfu/ml. Suspensions were swabbed on MHA (9 cm plates, with 25 ml medium). The plates were air dried for 15–20 min, and filter paper discs (6 mm diameter; Bioanalyse, Turkey) containing antibiotics were placed onto the inoculated agar. The plates were incubated at 37 °C for 24 h. The next day, the inhibition zone diameters were measured in millimeters and evaluated according to the CLSI breakpoint tables.

Biofilm formation

The biofilm forming abilities of the isolates were investigated using the Crystal Violet (CV) staining method (Dosler, & Karaaslan, 2014; Peeters, Nelis, & Coenye, 2008). Bacteria were adjusted with tryptic soy broth (TSB) glucose to a final concentration of approximately 1×10^7 cfu/ml. For the biofilm formation, the cell suspensions were placed into the wells of the microtiter plates (Greiner Bio-One, Kremsmuenster, Austria) and incubated for 24 h at 37°C. The next day, the remaining medium was aspirated gently, and the non-adherent cells were removed. For the biofilm fixations, 100 µl 99% of methanol was added to the wells, left to wait for 15 min, and aspirated; then the plates were air-dried. Next, the wells were stained with 100 µl 0.1% CV for 5 min, after which the excess CV was removed by washing the plates with tap water. The bound CV was solubilized by adding 95% ethanol over 30 min. Optical density (OD) was measured at 600 nm. For each isolate, biofilm formation was measured in triplicate, and *P. aeruginosa* PAO1 was used as a strong biofilm producer. Biofilm formation (weak, moderate, and strong) was interpreted as follows.

OD (isolate) ≤ OD (negative control) = negative biofilm formation;

OD (negative control) ≤ OD (isolate) ≤ 2xOD (negative control) = weak biofilm formation;

2xOD (negative control) ≤ OD (isolate) ≤ 4xOD (negative control) = moderate biofilm formation;

4xOD (negative control) ≤ OD (isolate) = Strong biofilm formation (Nirwati et al., 2019).

RESULTS

Disc diffusion assay

Table 1 summarizes the antibiotic disc diffusion susceptibility results, according to which most of the isolates were found resistant to carbapenems. More than 90% of the *A. baumannii*, *K. pneumoniae*, and *E. coli* isolates were found to be resistant to ceftazidime and piperacillin. Ciprofloxacin and piperacillin were ineffective against *E. coli*. Most of the *P. aeruginosa* isolates (75%) were found to be resistant to imipenem and aztreonam. Apart from aztreonam against *Serratia* sp. and gentamicin and amikacin against *Enterobacter* sp., all antibiotics were found ineffective.

Biofilm formation assay

All the isolates demonstrated the ability to form biofilms. Strong biofilm formation was observed in most of the strains except *K. pneumoniae*. Weak biofilm formation was only observed in five strains of *A. baumannii* (13.51%), nine strains of *K. pneumoniae* (26.47%), and one strain of *S. maltophilia* (25%; Table 2).

DISCUSSION

Gram-negative bacteria, most commonly *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *A. baumannii*, and *Enterobacter spp.* are responsible for causing various diseases, including bloodstream infections, urinary tract infections, pneumonia, wound or surgical site infections, and nosocomial infections (Dumaru, Baral, R., & Shrestha, et al., 2019). Antimicrobial resistance for Gram-negative bacteria is a growing global health threat. The World Health Organization (WHO) published a list of bacteria (grouped by priority as critical, high, and medium) that urgently need novel antibiotics in order to be fought, with most of these being Gram-negative bacteria (Breijyeh, Jubeh, & Karaman, 2020).

This study has investigated the antimicrobial resistance patterns of 98 Gram-negative bacteria. According to the results, most of the *A. baumanni* isolates were found to be resistant to all the studied antibiotics, even carbapenems. Carbapenems are one of the most-used and effective antibiotics against Gram-negative bacteria due to their broad-spectrum antibacterial activity that targets penicillin-binding proteins while

Table 1. Antibiotic resistance patterns of bacteria.

	A. baumannii (n=37)	*K. pneumoniae* (n=34)	*P. aeruginosa* (n=16)	*E. coli* (n=5)	*S. maltophilia* (n=4)	*Serratia sp.* (n=1)	*Enterobacter* sp. (n=1)
IPM	81	29	75	60	*	100	100
MEM	89	79	68	40	*	100	100
GN	91	79	56	20	*	100	0
TM	78	82	50	60	*	100	100
AN	91	76	50	20	*	100	0
CIP	94	85	56	100	*	100	100
LVX	83	85	50	100	50	100	100
CAZ	91	97	50	100	*	100	100
FEP	91	85	37	100	*	100	100
PIP	97	94	50	100	*	100	100
ATM	*	88	75	100	*	0	100

* Antibiotics not tested/not recommended by CLSI
Imipenem(IPM), meropenem (MEM), gentamicin (GN), tobramycin (TM), amikacin (AN), ciprofloxacin (CIP), levofloxacin (LVX), ceftazidime (CAZ), cefepime (FEP), piperacillin (PIP) and aztreonam (ATM)

Table 2. Biofilm formation rates of isolates.

	Strong	Moderate	Weak
A. baumannii (n=37)	65% (n=24)	22% (n=8)	13% (n=5)
K. pneumoniae (n=34)	29% (n=10)	44% (n=15)	26% (n=9)
P. aeruginosa (n=16)	81% (n=13)	18% (n=3)	-
E. coli (n=5)	60% (n=3)	40% (n=2)	-
S. maltophilia (n=4)	75% (n=3)	-	25% (n=1)
Serratia sp. (n=1)	100% (n=1)	-	-
Enterobacter sp. (n=1)	100% (n=1)	-	-
Total (n=98)	56% (n=55)	28% (n=28)	15% (n=15)

being relatively resistant to hydrolysis by most β-lactamases (El-Gamal et al., 2017). Infections caused by the multidrug-resistant *A. baumannii* strains are one of the most troublesome infections to treat because most clinical strains resistant to carbapenems are generally resistant to all classes of β-lactams as well as other classes of antibiotics (Malone & Kwon, 2013). Resistance rates are increasing all over the world, with 40%-70% of the strains being shown to be carbapenem resistant in infections acquired in intensive care units. The pattern of antibiotic resistance for *A. baumannii* in this study is similar to those found in many previous studies (Namaei et al., 2021, Yadav, Bhujel, & Mishra, 2020).

K. pneumoniae is another major threat to public health and the most common cause of hospital- and community-acquired infections, with carbapenem-resistant strains having been reported recently (Candan & Aksöz 2015). Among the antibiotics tested in this study, 71% of *K. pneumoniae* strains were susceptible to imipenem, while other antibiotics were mostly ineffective. Although some studies reported a lower resistance rate in *K. pneumoniae* against antibiotics (Dumaru et al., 2019; Cepas et al. 2019), resistance has gradually been increasing globally, similar to what this study found.

P. aeruginosa is an important pathogen that is able to cause bloodstream infections, surgical site infections, and lower respiratory system infections, especially in cystic fibrosis and immunocompromised patients. It is resistant to a variety of antibiotics, including carbapenems, aminoglycosides, quinolones, and β-lactams (Pang, Raudonis, Glick, Lin, & Cheng, 2019). Most of the *P. aeruginosa* isolates in this study were found to be resistant to carbapenems and aztreonam, with 37% of them being susceptible to cefepime. Cefepime is a fourth-generation cephalosporin and shows bactericidal activity by binding to penicillin-binding proteins and inhibiting peptidoglycan synthesis. Cefepime is widely used for the treatment of moderate-to-severe infections, including infections caused by *P. aeruginosa* (Jia et al., 2020). A similar pattern of cefepime resistance was also reported by Dumaru et al. (2019) for *P. aeruginosa*.

The current study found ciprofloxacin and piperacillin to be ineffective against the *E. coli* isolates, with all the *E. coli* strains also being found resistant to levofloxacin, ceftazidime, cefepime, and aztreonam. Only one *E. coli* isolate was found to be resistant to amikacin and gentamicin. Despite not being widely used due to concerns of toxicity, aminoglycosides including amikacin and gentamicin are still important therapeutic options for treating serious infections caused by Gram-negative bacteria (Bader, Loeb, Leto, & Brooks, 2020). A few recent studies in Turkiye on *E. coli* have shown amikacin and gentamicin to be effective against it, similar to this study's results (Mirza & Sancak 2020; Avcıoğlu & Behçet, 2020; İnce et al., 2021).

S. maltophilia is an important pathogen that primarily causes respiratory tract infections such as pneumonia and acute exacerbations of chronic obstructive pulmonary disease. The antibiotic options for treating *S. maltophilia* infections are limited due to its intrinsic resistance to a wide variety of antibiotics, including aminoglycosides, most β-lactams, and tetracyclines (Mojica et al., 2022). According to the current study's results, two of the four isolates were found to be susceptible to levofloxacin. Chung et al. (2012) studied the antimicrobial susceptibility of 90 clinical isolates of *S. maltophilia* and also showed levofloxacin to be effective against most of the isolates tested.

Despite the limited number of *Serratia sp.* and *Enterobacter sp.* strains studied here, aztreonam was found to be effective against *Serratia sp.* and amikacin against *Enterobacter sp.*

According to the National Institutes of Health, biofilms are a complex structure comprising microbial cells and extracellular matrix and are estimated to be responsible for 65% of all microbial infections and 80% of chronic infections (Jamal et al., 2018). Biofilm infections include non-device and device-associated infections such as central venous catheters, breast implants, urinary catheters, mechanical heart valves, peritoneal dialysis catheters, ventricular shunts, prosthetic joints, and contact lenses (Jamal et al., 2018).

Overall, 56.12% of the isolates were detected as strong and 28.57% as moderate biofilm formers in this study. These results are consistent with those from Dumaru et al.'s (2019) study, who detected 62.73% of the Gram-negative isolates, which included *E. coli*, *Acinetobacter sp.*, *Klebsiella sp.*, and *Pseudomonas* sp., to be biofilm positive. Similarly, Cepas et al. (2018) found a total of 49.3% of the *E. coli*, *K. pneumoniae*, and *P. aeruginosa* isolates to have the ability to form biofilms at respective rates of 30.3%, 37.6%, and 76.5%.

Biofilm formation is known to represent a conserved growth mode that makes bacteria less susceptible to antibiotics and unable to be killed by host immune mechanisms (Del Pozo, 2018). Antibiotic treatments may not be effective once the biofilm has matured (Jamal et al., 2018), so the high rate of biofilm formation in our strains may result in antibiotic resistance in addition to treatment failure.

CONCLUSION

To conclude, Gram-negative bacteria, most commonly *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii*, are mostly multi-drug resistant and becoming increasingly resistant to all available antibiotics. Antimicrobial resistance is a significant global problem, and WHO has declared it to be one of the top 10 global public health threats facing humanity. Therefore, surveilling Gram-negative bacteria's susceptibility profiles and biofilm formation is important because knowing the variable susceptibility patterns can aid in the appropriate management of the infections they cause.

Peer-review: Externally peer-reviewed.

Informed Consent: Written consent was obtained from the participants.

Author Contributions: Conception/Design of Study- M.H., F.N.Y. O.O.; Data Acquisition- M.H., F.N.Y. O.O., N.I.; Data Analysis/Interpretation- M.H., A.S.B.T.; Drafting Manuscript- M.H., F.N.Y. O.O.; Critical Revision of Manuscript- M.H., O.O., N.I., A.S.B.T.; Final Approval and Accountability- M.H., F.N.Y., O.O., N.I., A.S.B.T.

Conflict of Interest: The authors have no conflict of interest to declare

Financial Disclosure: This work was supported by the Research Fund of Istanbul University [project number 37825].

REFERENCES

- Avcıoğlu, F. & Behçet, M. (2020). Üriner sistem enfeksiyonu etkeni *Escherichia coli* izolatlarının çeşitli antibiyotiklere direnç oranlarının değerlendirilmesi. *Türk Mikrobiyoloji Cemiyeti Dergisi*, *50*(3), 172-7.
- Bader, M. S., Loeb, M., Leto, D., & Brooks, A. A. (2020). Treatment of urinary tract infections in the era of antimicrobial resistance and new antimicrobial agents. *Postgraduate Medicine*, *132*(3), 234–250. https://doi.org/10.1080/00325481.2019.1680052
- Breijyeh, Z., Jubeh, B., & Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules*, *25*(6), 1340.
- Candan, E. D., & Aksöz, N. (2015). *Klebsiella pneumoniae*: characteristics of carbapenem resistance and virulence factors. *Acta Biochimica Polonica*, *62*(4), 867–874. https://doi.org/10.18388/abp.2015_1148
- Center for Disease Control and Prevention (CDC). Antibiotic Resistance Threats in the United States, 2019. Atlanta, GA: U.S. Department of Health and Human Services, CDC; 2019.
- Cepas, V., López, Y., Muñoz, E., Rolo, D., Ardanuy, C., Martí, S., Xercavins, M., Horcajada, J. P., Bosch, J., & Soto, S. M. (2019). Relationship between biofilm formation and antimicrobial resistance in Gram-negative bacteria. *Microbial Drug Resistance* (Larchmont, N.Y.), *25*(1), 72–79. https://doi.org/10.1089/mdr.2018.0027
- Chung, H.S., Hong, SG., Lee, Y., Kim, M., Yong, D. Jeong, SH., Lee, K. & Chong, Y. (2012). Antimicrobial susceptibility of *Stenotrophomonas maltophilia* isolates from a Korean tertiary care hospital. *Yonsei Medical Journal 53*(2), 439-41. doi: 10.3349/ymj.2012.53.2.439. PMID: 22318836; PMCID: PMC3282950.
- Clinical and Laboratory Standards Institute (CLSI) (2021) Performance Standards for Antimicrobial. Document M100-Ed.31., Wayne, PA: CLSI.
- Del Pozo J. L. (2018). Biofilm-related disease. *Expert Review of Anti-Infective Therapy*, *16*(1), 51–65. https://doi.org/10.1080/14787210.2018.1417036
- Dosler, S. & Karaaslan E. (2014). Inhibition and destruction of *Pseudomonas aeruginosa* biofilms by antibiotics and antimicrobial peptides. *Peptides*, *62*, 32-37.
- Dumaru, R., Baral, R., & Shrestha, L. B. (2019). Study of biofilm formation and antibiotic resistance pattern of gram-negative Bacilli among the clinical isolates at BPKIHS, Dharan. *BMC Research Notes*, *12*(1), 38. https://doi.org/10.1186/s13104-019-4084-8
- Eichenberger, E. M., & Thaden, J. T. (2019). Epidemiology and mechanisms of resistance of extensively drug resistant Gram-negative bacteria. *Antibiotics, 8*(2), 37.
- El-Gamal, M. I., Brahim, I., Hisham, N., Aladdin, R., Mohammed, H., & Bahaaeldin, A. (2017). Recent updates of carbapenem antibiotics. *European Journal of Medicinal Chemistry*, *131*, 185–195. https://doi.org/10.1016/j.ejmech.2017.03.022
- Hammoudi Halat, D., & Ayoub Moubareck, C. (2020). The current burden of carbapenemases: Review of significant properties and dissemination among Gram-negative bacteria. *Antibiotics, 9*(4), 186.
- İnce, G., Mirza, H. C., Üsküdar Güçlü, A., Gümüş, H., Erol, Ç., & Başustaoğlu, A. (2021). Comparison of in vitro activities of plazomicin and other aminoglycosides against clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli*. *The Journal of Antimicrobial Chemotherapy*, *76*(12), 3192–3196. https://doi.org/10.1093/jac/dkab331
- Jamal, M., Ahmad, W., Andleeb, S., Jalil, F., Imran, M., Nawaz, M. A., Hussain, T., Ali, M., Rafiq, M., & Kamil, M. A. (2018). Bacterial biofilm and associated infections. *Journal of the Chinese Medical Association: JCMA*, *81*(1), 7–11. https://doi.org/10.1016/j.jcma.2017.07.012
- Jia, X., Ma, W., He, J., Tian, X., Liu, H., Zou, H., & Cheng, S. (2020). Heteroresistance to cefepime in *Pseudomonas aeruginosa* bacteraemia. *International Journal of Antimicrobial Agents, 55*(3), 105832. https://doi.org/10.1016/j.ijantimicag.2019.10.013
- Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., & Monnet, D. L. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical Microbiology and Infection, 18*(3), 268-281.
- Malone, L., & Kwon, D. H. (2013). Carbapenem-associated multidrug-resistant Acinetobacter baumannii are sensitised by aztreonam in combination with polyamines. *International journal of Antimicrobial Agents*, *41*(1), 70–74. https://doi.org/10.1016/j.ijantimicag.2012.08.009
- Mirza, H. C., Sancak, B., (2020). Comparison of antimicrobial susceptibilities of *Escherichia coli* isolated from urinary cultures of different patient groups: A university hospital experience. *Klimik Dergisi*, *33*(3), 270-6.
- Mojica, M. F., Humphries, R., Lipuma, J. J., Mathers, A. J., Rao, G. G., Shelburne, S. A., Fouts, D. E., Van Duin, D., & Bonomo, R. A. (2022). Clinical challenges treating *Stenotrophomonas maltophilia* infections: an update. *JAC-Antimicrobial Resistance*, *4*(3), dlac040. https://doi.org/10.1093/jacamr/dlac040
- Namaei, M. H., Yousefi, M., Askari, P., Roshanravan, B., Hashemi, A., & Rezaei, Y. (2021). High prevalence of multidrug-resistant non-fermentative Gram-negative bacilli harboring blaIMP-1 and blaVIM-1 metallo-beta-lactamase genes in Birjand, south-east Iran. *Iranian Journal of Microbiology*, 13(4), 470–479. https://doi.org/10.18502/ijm.v13i4.6971
- Nirwati, H., Sinanjung, K., Fahrunissa, F., Wijaya, F., Napitupulu, S., Hati, V. P., Hakim, M. S., Meliala, A., Aman A. T., & Nuryastuti, T. (2019). Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. *BMC Proceedings*, *13*(11), 1-8. https://doi.org/10.1186/s12919-019-0176-7
- Pang, Z., Raudonis, R., Glick, B. R., Lin, T. J., & Cheng, Z. (2019). Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and alternative therapeutic strategies. *Biotechnology Advances*, *37*(1), 177–192. https://doi.org/10.1016/j.biotechadv.2018.11.013
- Peeters, E., Nelis, H. J. & Coenye. T., (2008). Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *Journal of Microbiology Methods, 72*(2), 157-165. doi:10.1016/j.mimet.2007.11.010
- Perdikouri, E. I. A., Arvaniti, K., Lathyris, D., Apostolidou Kiouti, F., Siskou, E., Haidich, A. B., & Papandreou, C. (2019). Infections due to multidrug-resistant bacteria in oncological patients: insights from a five-year epidemiological and clinical analysis. *Microorganisms*, *7*(9), 277.
- Peters, L., Olson, L., Khu, D. T., Linnros, S., Le, N. K., Hanberger, H. & Larsson, M. (2019). Multiple antibiotic resistance as a risk factor for mortality and prolonged hospital stay: a cohort study among neonatal intensive care patients with hospital-acquired infections caused by Gram-negative bacteria in Vietnam. *PloS one*, *14*(5), e0215666.
- Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *The Journal of Infectious Diseases*, *197*(8), 1079-1081.
- Thaden, J. T., Li, Y., Ruffin, F., Maskarinec, S. A., Hill-Rorie, J. M., Wanda, L. C., & Fowler Jr, V. G. (2017). Increased costs associated with bloodstream infections caused by multidrug-resistant gram-negative bacteria are due primarily to patients with hospital-acquired infections. *Antimicrobial Agents and Chemotherapy, 61*(3), e01709-16.

- Vivas, R., Barbosa, A. A. T., Dolabela, S. S., & Jain, S. (2019). Multidrug-resistant bacteria and alternative methods to control them: an overview. *Microbial Drug Resistance*, *25*(6), 890-908.
- Wang, G., Zhao, G., Chao, X., Xie, L., & Wang, H. (2020). The characteristic of virulence, biofilm and antibiotic resistance of *Klebsiella pneumoniae*. *International Journal of Environmental Research and Public Health*, *17*(17), 6278.
- World Health Organization (WHO) https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance
- Yadav, S.K., Bhujel, R. & Mishra, S.K. (2020). Emergence of multi-drug-resistant non-fermentative gram negative bacterial infection in hospitalized patients in a tertiary care center of Nepal. *BMC Res Notes, 13*, 319. https://doi.org/10.1186/s13104-020-05163-6