IRSTI 31.23.25 https://doi.org/10.26577/ijbch.2022.v15.i1.05

E. Altınöz*, E.M. Altuner

Kastamonu University, Kastamonu, Turkey
*e-mail: altinozedaa@gmail.com

(Received 2 March 2021; received in revised form 30 March 2022; accepted 13 May 2022)

Observing the presence of efflux pump activities in some clinically isolated bacterial strains

Abstract. Chemotherapeutic agents are substances that cure infectious diseases for many years. However, problems arose due to drug resistance that developed over time and it was observed that the drugs failed to kill the bacteria. Studies have been carried out to find the factor caused by drug resistance that adversely affect the disease treatment, and these studies continue today. There are different types of resistance mechanisms. Efflux pumps are just one of these mechanisms. Efflux pumps cannot enter and release the drug into the cell. Thus, the microorganism becomes resistant to the drug used. If this resistance is inhibited, the drug may work. A variety of chemical or natural inhibitors are available for inhibition. However, since they cause toxicity problems, their clinical use is not currently available. Studies in this area are ongoing. There are studies to determine the activity of the efflux pump. The activity of this mechanism can be detected with ethidium bromide (EtBr). In this study, it was aimed to determine the efflux pump activity of some resistant clinically isolated strains using EtBr dye. The strains that are *Acinetobacter baumannii*, *Candida albicans*, *Candida glabrata*, *Candida tropicalis*, *Klebsiella pneumoniae*, *Providencia rustigianii*, *Serratia odorifera*, *Shigella flexneri*, *Staphylococcus aureus* and *Streptococcus pneumoniae* microorganisms. Cartwheel method was applied on agars containing TSB with EtBr with different concentrations (0.0 mg/L, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L and 2.5 mg/L). After the incubation, activation case was observed under UV light. It is concluded that each strain used has efflux pump activity. EtBr was released at 0.5 mg/L and did not fluoresce. Difference in fluorescence was observed under EtBr UV between 1.0 mg/L and 2.5 mg /L.
Key words: Drug resistance, efflux pump activities, clinically isolated bacterial strains, EtBr, inhibitors, UV.

Introduction

Microbial multidrug resistance (MDR) has reached a very important stage. As a result, the researches to discover new antibiotic hit compounds are accelerated. MDR efflux pumps mechanism is one of the important phenomena for antibiotic resistance.

Gram negative bacteria also have more antibacterial resistance than gram positive bacteria because gram negative bacteria have an outer membrane in addition to the cell wall structure [1-3].

Efflux pumps are transport proteins that play a role in expelling substances from inside towards the outside of the cells [4]. It can be present in all types of living cells [5].

The first exploration of the efflux pump mechanism was the excretion of tetracycline in *Escherichia coli* bacteria identified by Levy et al. [6-9].

There are 5 superfamilies defined for the efflux pumps, which are the ATP Binding Cassette (ABC), Multidrug and Toxic Efflux (MATE), Major Facilitator Superfamily (MFS), Resistance Nodulation Division (RND) and Small Multidrug Resistance (SMR) [4, 10, 11]. In addition to these, The Proteobacterial Antimicrobial Compound Efflux (PACE) family was also defined in the study published by Du et al. [12] and the Drug Metabolite Transporter (DMT) family by Song and Wu [13]. DMT is a large group of membrane transporters present in eukaryotes, bacteria and archaea, and includes exporters for a wide range of substrates such as toxic compounds and metabolites [13-15].

As a result of inhibiting efflux pumps, the antimicrobial compounds can accumulate inside the cell, which may cause a cell death. Although there are some compounds that inhibit efflux pumps, but they are not commonly used clinically due to their toxicity [16].

Efflux pump inhibitors can act on either gram positive or gram-negative bacteria or both [17]. The examples of some efflux pump inhibitors and their mode of action are as follows.

Verapamil inhibits ABC/MFS family, Thioridazine inhibits NorA pump of the MFS family, Carbonyl Cyanide m-Chlorophenyl Hydrazone (CCCP) inhibits MATE/MFS/RND family, Phenylalanyl Arginine Beta Naphtylamid (PAβN/ MC-207, 110) inhibits RND/MFS (CmIA and FloR pump) family, Paroxetine inhibits RND/MFS family and Reserpine inhibits RND/MFS family [10, 13, 18-33].

The aim of this study is observing the presence of efflux pump activity in some multidrug resistant (MDR) clinically isolated bacterial strains to define their efflux pump activity profile.

Materials and methods

Enrichment of microorganisms and isolation of pure colonies. In this study, totally 10 clinical isolate MDR strains were used. 7 of the strains are bacteria (*Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Providencia rustigianii*, *Serratia odorifera*, *Shigella flexneri*, *Staphylococcus aureus* and *Streptococcus pneumoniae*) and 3 are yeast (*Candida albicans*, *Candida glabrata* and *Candida tropicalis*). Microorganisms were activated Luria-Bertani (LB) Broth (Merck, Germany) and then transferred to Nutrient Agar (NA) (Merck, Germany) for pure colony formation. Pure colonies obtained in NA were transferred into sterile saline solution (0.9%) and standardized by adjusting according to 0.5 McFarland standards.

Ethidium bromide (EtBr)-Tryptic Soy Agar (TSB) cartwheel method. TSB (Merck, Germany) plates, which included different EtBr (Merck, Germany) concentrations (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/L) were prepared. Microorganisms were inoculated on the plates according the cartwheel method. Cartwheel methodology was used (Figure 1) [34]. Then the petri dishes were incubated at 37°C for 24 hours for bacterial strains and 27°C for 48 hours for yeasts.

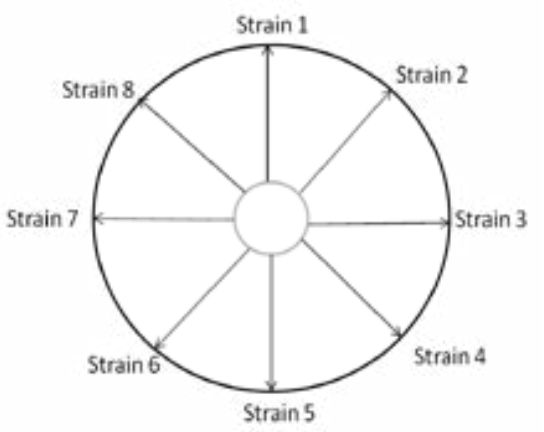

Figure 1 – Cartwheel method

Observing under UV light. After incubation, TSB agar plates containing EtBr were observed under UV light.

Statistical analysis. R Studio, version 4.0.2 was used to conduct a one-way analysis of variance (ANOVA) to analyze the results (P = 0.05) [35].

Results and discussion

In order to obtain results, plates were observed under UV light. A fluorescence was observed at different concentrations of EtBr for each microorganism under UV light on TSB agar plates. 0.0 mg/L concentration is the negative control group. Absence of fluorescence was expected as EtBr was absent at 0.0 mg/L. Therefore, no fluorescence was observed for these plates (0.0 mg/L), presented on Figure 2.

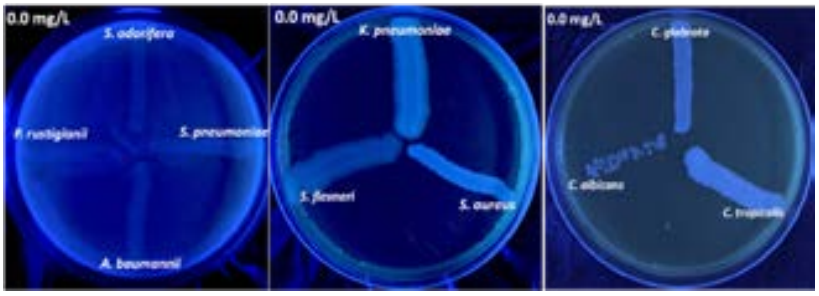

Figure 2 – UV fluorescence for 0.0 mg/L EtBr concentration

Since the amount of EtBr dye at 0.5 mg/L was too low, microorganisms were expected to efflux EtBr out and the results confirmed this, presented on Figure 3.

For 1.0 mg/L of EtBr concentration; as the concentration started to increase, the majority of EtBr began to remain in the cell and a purple fluorescence started to appear. A low fluorescence for 4 microorganisms (*S. odorifera*, *S. pneumoniae*, *A. baumannii*, *P. rustigianii*) was observed in the first plate. Differences in purple fluorescence of microorganisms were observed on the second plate and the purple fluorescence of *C. albicans* in the third plate is higher than that for *C. glabrata* and *C. tropicalis*. Results presented on Figure 4.

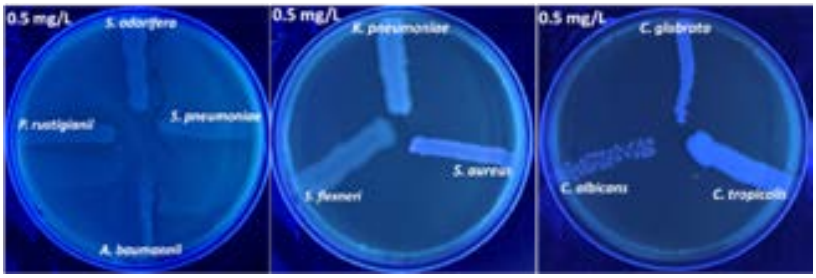

Figure 3 – UV fluorescence for 0.5 mg/L EtBr concentration

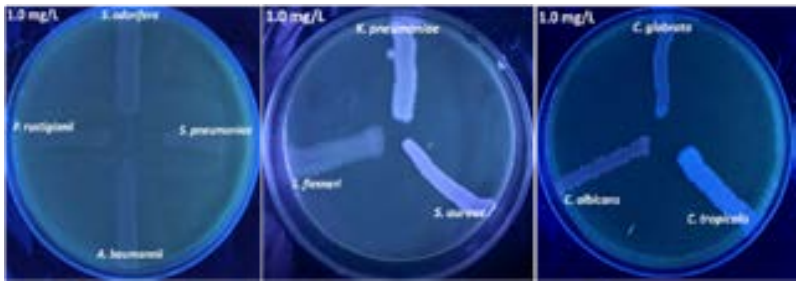

Figure 4 – UV fluorescence for 1.0 mg/L EtBr concentration

For 1.5 mg/L of EtBr concentration; EtBr was started to accumulate in the cells of all microorganisms, although there are differences between them. Fluorescence of 4 microorganisms (*S. odorifera*, *S. pneumoniae*, *A. baumannii*, *P. rustigianii*) in the first plate was also observed to be equal. Differences in fluorescence of microorganisms were observed on the second plate. *S. aureus* and *K. pneumoniae* presented equal fluorescence and the purple fluorescence in *S. flexneri* was observed to be more intense. The fluorescence of *C. albicans* in the third plate was found to be higher than the others. Results presented on Figure 5.

For 2.0 mg/L of EtBr concentration; EtBr was accumulated in the cells of all microorganisms, although there are differences between them. Fluorescence of 4 microorganisms (*S. odorifera*, *S. pneumoniae*, *A. baumannii*, *P. rustigianii*) in the first plate was also observed to be equal. Differences in fluorescence for *S. aureus*, *K. pneumoniae* and *S. flexneri* were observed on the second plate. The fluorescence of *C. albicans* and *C. tropicalis* in the third plate was higher than *C. glabrata*. Results presented on Figure 6.

2.5 mg/L concentration is the highest concentration. EtBr was accumulated in the cells of all microorganisms. Because of the high EtBr concentration, the intensity of fluorescence was higher than other concentrations. Results presented on Figure 7.

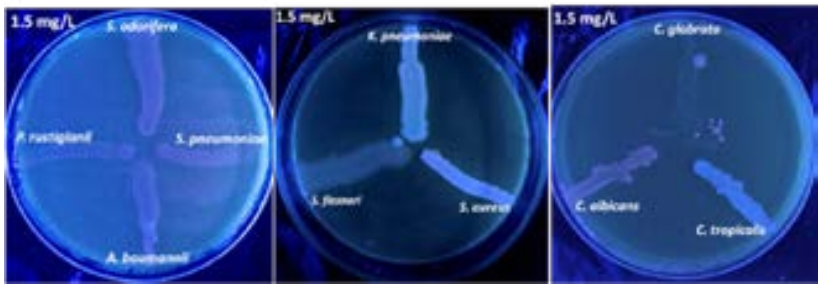

Figure 5 – UV fluorescence for 1.5 mg/L EtBr concentration

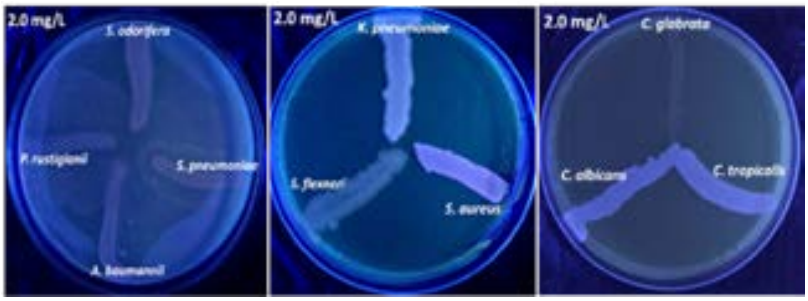

Figure 6 – UV fluorescence for 2.0 mg/L EtBr concentration

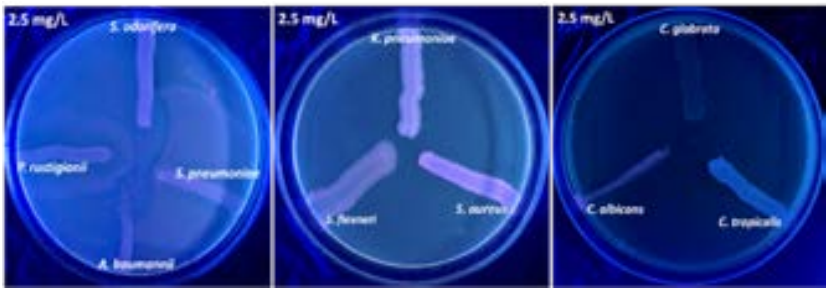

Figure 7 – UV fluorescence for 2.5 mg/L EtBr concentration

According to the results it was observed that the maximum EtBr concentration effluxed out the cell changes between 1.0 and 2.0 mg/L of EtBr for all strains used in the study. In all strains, the intensity of the fluorescence was increased, as the EtBr concentration was increased from 1.0 mg/L to 2.5 mg/L, where the highest fluorescence was at 2.5 mg/L concentration.

In a previous study, which was conducted by MDR *E. coli* strains, the activity of the efflux pumps against commercial efflux pump inhibitors were observed. Cartwheel test with EtBr was applied to microorganisms and similar results were observed under UV light. In addition, verapamil, thioridazine hydrochloride and phenyl arginine beta naphthylamide (PAβN) inhibitors were tested in combination with some antibiotics for inhibiting bacterial growth and positive results were obtained [11].

In a study by Anbazhagan et al. MDR *Salmonella Typhimurium* strains were used and the efflux pump activity was evaluated. They applied the EtBr cartwheel method in the experiment. They prepared MH agar plates with EtBr containing 0.0 mg/L, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L and 2.0 mg/L. After the incubation, the plates were examined under UV and their fluorescence were observed. It was reported that the efflux activities of 1.0 mg/L and 1.5 mg/L

EtBr on strains are well differentiated. They observed the efflux pump activity of strains by using PAβN inhibitor and some antibiotics. It was stated that 8 out of 9 strains they used in the study showed efflux pump activity and at least one antibiotic had an effect [36]. In the study by Martins et al. MDR *E. coli*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Enterococcus faecalis*, *Enterobacter aerogenes* and *Staphylococcus aureus* bacteria strains were used. They applied the cartwheel method in TSB agars with EtBr at concentrations between 0.0 mg/L and 2.5 mg/L to observe the presence of efflux pumps [34]. Jiang et al. used the LightCycler 96 instrument (Roche, Basel, Switzerland) to measure (533 and 572 nm) the fluorescence of EtBr in the cell in their study. Thus, they studied the role of efflux pump in ciprofloxacin resistance of *Listeria monocytogenes* they used [37].

Conclusion

As stated in other studies, after the efflux pump activity was determined, inhibitor studies were performed with antibiotic combinations. Studies should continue to discover a non-toxic inhibitor and to investigate which efflux pump the inhibitor acts on. Thus, it is thought that it may overcome the major problem of antibiotic resistance. In this study, efflux pump activity was observed in drug resistant strains. Each strain used had efflux pump activity. Especially, different fluorescence was observed under EtBr UV between 1.0 mg/L and 2.5 mg/L. It is clear that, researches about efflux pumps have an extreme importance since they are important in bacterial resistance and this study clearly presents that each strain could have different efflux capacities, so it is important to observe the efflux pump activities of each microorganism before conducting efflux pump inhibition researches. Various results can be obtained by observing efflux pump activity by working with more various microorganisms.

References

1. Gan L., Chen S., Jensen G. (2008) Molecular organization of Gram-negative peptidoglycan. *Proc Natl Acad Sci U S A*., vol. 105, no. 48, pp. 18953–18957. https://doi.org/10.1021/jo035397e.s001.

2. Vollmer W., Blanot D., De Pedro M.A. (2008) Peptidoglycan structure and architecture. *FEMS Microbiol. Rev*., vol. 32, no. 2, pp. 149-167. https://doi.org/10.1111/j.1574-6976.2007.00094.x.

3. Altınöz E., Altuner E.M. (2019) Antibiotic Resistance and Efflux Pumps. *Int. J. Inn. Res. Rev*., vol. 3, no. 2, pp. 1-9.

4. Webber M.A., Piddock L.J.V. (2003) The importance of efflux pumps in bacterial antibiotic resistance. *J. Antimicrob. Chemother*., vol. 51, no. 1, pp. 9-11. https://doi.org/10.1093/jac/dkg050.

5. Alcalde-Rico M., Hernando-Amado S., Blanco P., Martínez, J. L. (2016) Multidrug efflux pumps at the crossroad between antibiotic resistance and bacterial virulence., *Front. Microbiol*., vol. 7, no. 1483, pp. 1-14. https://doi.org/10.3389/fmicb.2016.01483.

6. McMurry L., Levy S.B. (1978) Two transport systems for tetracycline in sensitive Escherichia coli: critical role for an initial rapid uptake system insensitive to energy inhibitors. Antimicrob Agents Chemother., vol. 14, no. 2, pp. 201-209. https://doi.org/10.1128/aac.14.2.201.

7. McMurry L., Petrucci R.E., Levy S.B. (1980) Active efflux of tetracycline encoded by four genetically different tetracycline resistance determinants in Escherichia coli. *Proc Natl Acad Sci U S A*., vol. 77, no. 7, pp. 3974-3977. https://doi.org/10.1073/pnas.77.7.3974.

8. Nelson M.L., Levy S.B. (2011) The history of the tetracyclines. *Ann N Y Acad Sci.*, vol. 1241, no. 1, pp. 17-32. https://doi.org/10.1111/j.1749-6632.2011.06354.x.

9. Kumar S., Varela, M.F. (2013) Molecular mechanisms of bacterial resistance to antimicrobial agents. In A. Méndez-Vilas (Ed.), Microbial Pathogens and Strategies for Combating Them: Science, Technology and Education 522-534 p. Badajoz:Formatex Research Center.

10. Lomovskaya O., Warren M.S., Lee, A., et al. (2001) Identification and characterization of inhibitors of multidrug resistance efflux pumps in Pseudomonas aeruginosa: novel agents for combination therapy. *Antimicrob Agents Chemother*., vol. 45, no. 1, pp. 105-116. https:// doi.org/10.1128/AAC.45.1.105-116.2001.

11. Altınöz E., Altuner E.M. (2020) Responses of some Escherichia coli clinical isolate strains with multiple drug resistance and overexpressed efux pumps against efux pump inhibitors. International Journal of Biology and Chemistry, vol. 13, no. 1, pp. 77-87. https:/doi.org/10.26577/ijbch.2020.v13.i1.08.

12. Du D., Wang-Kan X., Neuberger A., van Veen H.W., Pos K.M., Piddock L.J., Luisi B.F. (2018) Multidrug efflux pumps: structure, function and regulation. *Nat Rev Microbiol.*, vol. 16, no. 9, pp. 523-539. https://doi.org/10.1038/s41579-018-0048-6.

13. Song L., Wu X. (2016) Development of efflux pump inhibitors in antituberculosis therapy. *Int J Antimicrob Agents.*, vol. 47, no. 6, pp. 421-429. https://doi.org/10.1016/j.ijantimicag.2016.04.007.

14. Jack D.L., Yang N.M., Saier M.H. (2001) The drug/metabolite transporter superfamily. *Eur J Biochem.*, vol. 268, no. 13, pp. 3620-3639. https://doi.org/10.1046/j.1432-1327.2001.02265.x

15. Tsuchiya H., Doki S., Takemoto M., et al. (2016) Structural basis for amino acid export by DMT superfamily transporter YddG. *Nature.*, vol. 534, no. 7607, pp. 417-420. https://doi.org/10.1038/nature17991.

16. Abdi S.N., Ghotaslou R., Ganbarov K., Mobed A., Tanomand A., Yousefi M., Asgharzadeh M., Kafil H.S. (2020) Acinetobacter baumannii Efflux Pumps and Antibiotic Resistance. *Infect Drug Resist.*, vol. 13, pp. 423-434. https:/doi.org/ 10.2147/IDR.S228089.

17. Kumar R., Pooja Patial S.J. (2016) A review on efflux pump inhibitors of gram-positive and gram-negative bacteria from plant sources. *Int J Curr Microbiol Appl Sci.*, vol. 5, no. 6, pp. 834-855. https://doi.org/10.20546/ijcmas.2016.506.092.

18. Van Bambeke F., Lee V.J. (2006) Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. *Recent Pat Antiinfect Drug Discov.*, vol. 1, no. 2, pp. 157-175. https:/doi.org/10.2174/157489106777452692.

19. Gupta A.K., Chauhan D.S., Srivastava K., et al.(2006) Estimation of efflux mediated multi-drug resistance and its correlation with expression levels of two major efflux pumps in mycobacteria. *J Infect Dis.*, vol. 38, no. 3, pp. 246-254.

20. Kristiansen M.M., Leandro C., Ordway D., et al. (2006) Thioridazine reduces resistance of methicillin-resistant Staphylococcus aureus by inhibiting a reserpine-sensitive efflux pump. *In vivo.*, vol. 20, no. 3, pp. 361-366.

21. Seeger M.A., Diederichs K., Eicher T., Brandstatter L., Schiefner A., Verrey F., Pos K.M. (2008) The AcrB efflux pump: conformational cycling and peristalsis lead to multidrug resistance. *Curr Drug Targets*, vol. 9, no. 9, pp. 729-749. https://doi.org/10.2174/138945008785747789.

22. Li X.Z., Nikaido H. (2009) Efflux-mediated drug resistance in bacteria. *Drugs.*, vol. 69, no. 12, pp. 1555-1623. https://doi.org/10.2165/11317030-000000000-00000.

23. Vecchione J.J., Alexander B., Sello, J.K. (2009) Two distinct major facilitator superfamily drug efflux pumps mediate chloramphenicol resistance in Streptomyces coelicolor. *Antimicrob Agents Chemother.*, vol. 53, no. 11, pp. 4673-4677. https://doi.org/10.1128/AAC.00853-09.

24. Gupta A.K., Reddy V.P., Lavania M., et al. (2010) jefA (Rv2459), a drug efflux gene in Mycobacterium tuberculosis confers resistance to isoniazid & ethambutol. Indian J Med Res, 132(2), 176-188.

25. Okandeji B.O., Greenwald D.M., Wroten J., Sello, J.K. (2011) Synthesis and evaluation of inhibitors of bacterial drug efflux pumps of the major facilitator superfamily. *Bioorg Med Chem*., vol. 19, no. 24, pp. 7679-7689. https://doi.org/10.1016/j.bmc.2011.10.011.

26. Tegos P.G., Haynes M., Strouse J.J., et al. (2011) Microbial efflux pump inhibition: tactics and strategies. *Curr Pharm Des*., vol. 17, no. 13, pp. 1291-1302. https://doi.org/10.2174/138161211795703726.

27. Rodrigues L., Machado D., Couto I., Amaral L., Viveiros, M. (2012) Contribution of efflux activity to isoniazid resistance in the Mycobacterium tuberculosis complex. *Infect Genet Evol*., vol. 12, no. 4, pp. 695-700. https://doi.org/10.1016/j.meegid.2011.08.009.

28. Rodrigues L., Villellas C., Bailo R., Viveiros M., Aínsa, J.A. (2013) Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. *Antimicrob Agents Chemother*., vol. 57, no. 2, pp. 751-757. https://doi.org/10.1128/AAC.01482-12.

29. Adams K.N., Szumowski J.D., Ramakrishnan, L. (2014) Verapamil, and its metabolite norverapamil, inhibit macrophage-induced, bacterial efflux pump-mediated tolerance to multiple anti-tubercular drugs. *J. Infect. Dis*., vol. 210, no. 3, pp. 456-466. https://doi.org/10.1093/infdis/jiu095.

30. Spengler G., Csonka Á., Molnár J., Amaral L. (2016) The anticancer activity of the old neuroleptic phenothiazine-type drug thioridazine. *Anticancer Res*., vol. 36, no. 11, pp. 5701-5706. https://doi.org/10.21873/anticanres.11153.

31. Singh G., Srivastava A.K. (2017) Efflux Pump Inhibitors: Enhance Therapy And Cauterize Tuberculosis. *Int J Pharm Sci Res*., vol. 8, no. 7, pp. 2762-2767. https://doi.org/10.13040/IJPSR.0975-8232.8(7).2762-67.

32. Lowrence R.C., Subramaniapillai S.G., Ulaganathan, V., et al. (2019) Tackling drug resistance with efflux pump inhibitors: from bacteria to cancerous cells. *Crit Rev Microbiol.*, vol. 45, no. 3, pp. 334-353. https://doi.org/10.1080/1040841X.2019.1607248.

33. Sharma A., Gupta V.K., Pathania R. 2019. Efflux pump inhibitors for bacterial pathogens: From bench to bedside. *Indian J Med Res*., vol. 149,

no. 2, pp. 129-145. https://doi.org/10.4103/ijmr.IJMR_2079_17.

34. Martins M., Couto I., Viveiros M., Amaral L. (2010) Identification of efflux-mediated multi-drug resistance in bacterial clinical isolates by two simple methods. In: Antibiotic resistance protocols. Humana Press, 143-157 p. https://doi.org/10.1007/978-1-60327-279-7_11.

35. Core R Team (accessed at December 15, 2020) "R: A language and environment for statistical computing. R Foundation for Statistical Computing." R-project org. https://www.R-project.org/.

36. Anbazhagan P.V., Thavitiki P.R., Varra M., Annamalai L., Putturu R., Lakkineni V.R., Pesingi P.K. (2019) Evaluation of efflux pump activity of multidrug-resistant Salmonella typhimurium isolated from poultry wet markets in India. *Infect Drug Resist.*, vol. 12, pp. 1081-1088. https://doi.org/1081.10.2147/IDR.S185081.

37. Jiang X., Yu T., Xu P., Xu X., Ji S., Gao W., Shi L. (2018) Role of efflux pumps in the in vitro development of ciprofloxacin resistance in Listeria monocytogenes. *Front. Microbiol.*, vol. 9, no. 2350, pp. 1-12. https://doi.org/10.3389/fmicb.2018.02350.