INFOBIOMAT

INTRAMAMMARY ETHNO-VETERINARY FORMULATION IN PREVENTION AND TREATMENT OF BOVINE MASTITIS FOR OPTIMIZATION OF ANTIMICROBIAL TREATMENT

PROCEEDINGS OF THE CONFERENCE

Antimicrobial Resistance in Veterinary Medicine - Current State and Perspectives

21-23. JUNE 2022.
NOVI SAD, SERBIA

POLJOPRIVREDNI FAKULTET
UNIVERZITET U NOVOM SADU

PROCEEDINGS OF THE CONFERENCE

Antimicrobial Resistance in Veterinary Medicine - Current State and Perspectives

21-23. JUNE 2022.
NOVI SAD, SERBIA

ISBN: 978-86-7520-555-5

Antimicrobial Resistance in Veterinary Medicine-Current State and Perspectives 2022

Book of Proceedings

Publisher
University of Novi Sad, Faculty of Agriculture
21000 Novi Sad, Trg D. Obradovića 8
Tel.:++(021) 6350-711; 4853-308;
polj.uns.ac.rs

On behalf of Publisher
Dr Nedeljko Tica, Full professor

President of the Scientific and Program board
Dr Zorana Kovačević, Associate professor

President of the Organizational board
Dr Mihajlo Erdeljan, Associate professor

Editors in Chief
Dr Zoran Ružić, Assistant professor
DVM Ivan Galić, Teaching assistant

Secretary
DVM Dragana Tomanić

Paper review
All papers reviewed by The International Board of Reviewers

Recorded by
Futura d.o.o.

Copies
200

HONORARY BOARD:

Marijana Dukić Mijatović / State Secretary of the Ministry of Education, Science and Technological Development
Danilo Golubović / Advisor - Ministry of European Integration
Milica Đurić Jovičić/ Director of the Science Fund of the Republic of Serbia
Nedeljko Tica / Dean of the Faculty of Agriculture, University of Novi Sad
Milorad Mirilović / Dean of the Faculty of Veterinary Medicine, University of Belgrade
Snežana Brkić / Dean of the Faculty of Medicine, University of Novi Sad
Ivan Stančić / Director of the Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad
Saša Bošković / Director of the Veterinary Office of Bosnia and Herzegovina
Vesna Đorđević / Director of the Institute of Meat Hygiene and Technology, Belgrade
Sava Lazić / Director of the Scientific Veterinary Institute Novi Sad
Darko Bošnjak / Director of the Veterinary Specialist Institute Zrenjanin
Mišo Kolarević / President of the Veterinary Chamber of Serbia
Ivan Zemljak / President of the Veterinary Chamber Of Croatia
Velibor Kesić / President of the Veterinary Chamber of Republika Srpska
Ranko Savić / President of the Veterinary Chamber of Montenegro
Tomislav Nikolovski / President of the Veterinary Chamoer of the Republic of Northern Macedonia
Dragica Stojanović / Faculty of Agriculture, University of Novi Sad

SCIENTIFIC AND PROGRAM BOARD:

President of the scientific and program board:
Zorana Kovačević / Faculty of Agriculture, University of Novi Sad

Peter Panduro Damborg / Department of Veterinary Medicine, University of Copenhagen
Els M. Broens / Faculty of Veterinary Medicine, University of Utrecht
Petra Cagnardi / Faculty of Veterinary Medicine, University of Milan
Frane Božić / Faculty of Veterinary Medicine, University of Zagreb
Iskra Cvetkovikj / Faculty of Veterinary Medicine, University of Skopje
Snežana Mugoša / Faculty of Medicine, University of Podgorica
Ahmed Smajlović / Faculty of Veterinary Medicine, University of Sarajevo
Drago Nedić / PI Veterinary Institute of the Republik of Srpska in Banja Luka
Bojan Toholj / Faculty of Agriculture, University of Novi Sad
Miodrag Radinović / Faculty of Agriculture, University of Novi Sad
Nebojša Kladar / Faculty of Medicine, University of Novi Sad

ORGANIZATIONAL BOARD:

President of the organizational board:
Mihajlo Erdeljan / Faculty of Agriculture, University of Novi Sad

Biljana Božin / Faculty of Medicine, University of Novi Sad
Ivana Ćirković / Faculty of Medicine, University of Belgrade
Aleksandra Kovačević / Military Medical Academy, Belgrade
Aleksandar Potkonjak / Faculty of Agriculture, University of Novi Sad
Dalibor Todorović / Scientific Veterinary Institute Novi Sad
Zoran Ružić / Faculty of Agriculture, University of Novi Sad
Ozren Smolec / Faculty of Veterinary Medicine, University of Zagreb
Ivana Davidov / Faculty of Agriculture, University of Novi Sad
Anamarija Galfi / Faculty of Agriculture, University of Novi Sad
Jovan Spasojević / Faculty of Agriculture, University of Novi Sad
Vuk Vračar / Faculty of Agriculture, University of Novi Sad
Tijana Kukurić / Faculty of Agriculture, University of Novi Sad
Ivan Galić / Faculty of Agriculture, University of Novi Sad
Jovan Stanojević / Faculty of Agriculture, University of Novi Sad

Conference organizer / Organizatori Konferencije:

Faculty of Agriculture, Department of Veterinary Medicine,
University of Novi Sad

Patron of the Conference / Pokrovitelji Konferencije:

Fond za nauku Republike Srbije

Veterinarska komora Srbije

Grad Novi Sad

Pokrajinski sekretarijat za poljoprivredu, vodoprivredu i šumarstvo

Garancijski Fond Autonomne Pokrajine Vojvodine

The COST Action CA18217 European Network for Optimization of Veterinary Antimicrobial Treatment (ENOVAT)

General sponsor of the Conference /
Generalni sponzor Konferencije:

NUTRITION FEIX FEED ADDITIVES

Conference sponsors / Sponzori Konferencije:

BIO-FLOPET d.o.o

VET NUTRITION

PROMEDIA
Laboratory supply specialists

Galen pharm

INSTITUT ZA HIGIJENU I TEHNOLOGIJU MESA • Beograd 1955 •

VETERINARSKI ZAVOD Subotica
a LABIANA Company

Gramma Libero

FRAME
MARKETING AGENCY

Cvejić Apoteka
Osnovana 1888.

KRKA

MIT
MEDICINSKI INSTRUMENTI I TEHNOLOGIJA
www.mit-med.co.rs

CONTENT

THE EFFECT OF ANTIBIOTIC USE IN FOOD-PRODUCING ANIMALS ON THE OCCURRENCE OF ANTIMICROBIAL RESISTANCE IN THE ENVIRONMENT

Petra P. Cagnardi[1*], Federica Di Cesare[1], Susanna Draghi[1], Alessandra Piccirillo[2], Andrea Laconi[2]

[1]*University of Milan, Department of Veterinary Medicine and Animal Sciences, Lodi, Italy*
[2]*University of Padua, Department of Comparative Biomedicine and Food Science, Padua, Italy*
[*]*e-mail: petra.caganrdi@unimi.it*

Abstract

The use of veterinary antibiotics in food-producing animals can contaminate the environment with consequent risks on health via resistant bacteria, on organic material and on the biotic environment. A research project was granted by the Italian Ministry of School Education, University and Research to evaluate the role of intensive animal farming, as potential source of environmental antimicrobial contamination and antimicrobial resistance. Samples of manure or slurry and soil (before and one month after fertilization) were collected from conventional chicken, swine and dairy farms. Analyses were carried out by LC-MS/MS to generate data on antimicrobial concentrations. Further analyses were carried out to generate data on antimicrobial resistance genes (ARGs) and microbial community composition of animal manure and soil before and after the application of manure by combining qPCR and 16S rRNA gene sequencing. Thirty-nine out of 93 samples were positive to at least one antimicrobial. Flumequine was the most detected, followed by enrofloxacin, tylosin, marbofloxacin, ampicillin and ciprofloxacin at a much lower prevalence. The most abundant *phyla* identified are those typical of manure and soil microbiomes. Manure application showed limited influence on soil microbial community and did not increase soil diversity. The highest number of total ARGs was detected in manure and an increase in ARGs abundance was observed in soil after fertilization. Flumequine may exert selective pressure for the accumulation of resistance genes in fertilized soil; hence, the use of flumequine in the veterinary field should be reconsidered. Swine farms were characterized by the highest abundance of ARGs.

Key words: antimicrobials, antimicrobial resistance genes, microbiome, manure application, agricultural soil, conventional animal farming

Introduction

The development of resistant bacteria strains is a possible consequence of incorrect and indiscriminate antimicrobial drugs (AMDs) use and this poses an alarming risk to human and animal health. The use of veterinary antibiotics in food-producing animals is linked with the enhanced selection of strains resistant to AMD used also in human medicine. Moreover, the use of veterinary and human AMDs can contaminate the environment with consequent risks, due to the indirect effect on health via resistant bacteria, the direct damage on organic material and the influences on the biotic environment (Zang et al., 2017).

Bovine, swine and poultry farms are a potential big source of environmental spread of contaminants and AMDs residues, as land application of manure is an effective method to dispose swine, bovine and poultry waste. Manure and waste slurries potentially contain significant amounts of AMD and their presence can persist in soil after land application (Zang et al., 2017).

Thus, the environmental consequences from the soil fertilization with drug-contaminated manure represent a topic of increasing interest and an emerging concern on the occurrence and spread of antimicrobial resistance (AMR) in the environment. The distribution of drug-contaminated manure can affect the composition and functional properties of microbial communities or microbiome and the presence of AMR can influence environmental resistome.

A research project was granted by the Italian Ministry of School Education, University and Research to evaluate the role of intensive animal farming (poultry, cattle and swine), as potential source of environmental antimicrobial contamination and resistance.

Materials and methods

Samples were collected from 31 conventional farms (10 chicken farms, 10 swine farms, and 11 dairy farms) located in two regions in Northern Italy. In each farm, three samples were collected from: 1) manure or slurry, and 2) soil (one sample before fertilization with manure/slurry and one sample one month after fertilization), accounting for a total of 93 samples (i.e. 3 samples x 31 farms). Analyses were carried out in order to generate data on antimicrobial concentrations, the analytical LC-MS/MS method was set up and validated in soil, manure and slurry to detect and quantify 14 different antimicrobials: amoxicillin, ampicillin, cefquinome, ceftiofur, ciprofloxacin, danofloxacin, enrofloxacin, flumequine, marbofloxacin, erythromycin, spiramycin, tilmicosin, tylosin and colistin. Further analyses were carried out in order to generate data on antimicrobial resistance genes (ARGs) and microbial community

composition of animal manure and soil before and after the application of manure by combining qPCR and 16S rRNA gene sequencing.

Results and discussion

Out of 93 samples, only 39 were found positive to at least one antimicrobial. Flumequine was the most detected (38/93 of the total samples), followed by enrofloxacin (7/93), tylosin (3/93), and marbofloxacin, ampicillin and ciprofloxacin (2/93) at a much lower prevalence. This is in agreement with flumequine known high persistence in manure, where it can remain after one year, thus its environmental presence was quite expected. A low percentage of positive samples (42%) was found in the study, and only six out of the 14 antimicrobials screened were detected.

The most abundant *phyla* identified are those typical of manure and soil microbiomes and the manure microbiome confirms to be less diverse than soil (Fierer, 2017; Hamm et al., 2016; Chen et al., 2015; Looft et al., 2012). In accordance with previous observations (Xie et al., 2018; Riber et al., 2014), manure application showed limited influence on soil microbial community and did not increase soil diversity, but rather caused significant changes only in a few *phyla*. These results might be due to the inability of most manure-associated bacteria to survive for long periods in soil, making the time of sampling a key factor and suggesting that manure microbiome might influence only temporarily the soil microbial community. Furthermore, factors other than manure application (i.e. temperature, moisture, pH, seasonality) are known to influence soil microbial composition over time, and might be accountable for such results (Fierer, 2017).

The highest number of total ARGs copies was detected in manure and a clear increase in ARGs abundance was observed in soil after fertilization with *ermA, ermB,* bla_{OXA-1} and *oqxA* being significantly enriched. According to previous studies, this finding indicates that manure enriched ARGs abundance in the soil, but none of the selected ARGs emerged in soil after manure application, suggesting that such practice might effectively enrich but not introduce any of the screened genes.

Not surprisingly, swine farms showed the highest total ARG abundance, and bla_{OXA-1}, *ermB*, *mcr-1* and *qnrS* were significantly more abundant in this sector in comparison to the others. Positive correlations between *oqxA* and *qnrS* abundances and flumequine concentrations were observed, together with the co-occurrence of some ARGs and microbial taxa.

Conclusion

In the study, it was demonstrated that fertilization may affect the abundance of specific ARGs in soil. The main conclusions were: a) manure-derived bacteria does not survive in soil, and manure application does not drastically affect the soil microbiome; b) different manure-derived AGRs experience different fates in soil; c) flumequine may exert selective pressure for the accumulation of resistance genes in fertilized soil; hence, the use of flumequine in the veterinary field should be reconsidered; d) the different dairy cattle, chicken and swine farms displayed different microbial communities, and swine farms were characterized by the highest abundance of ARGs.

Acknowledgements

This work was supported by the Italian Ministry of Research and University (PRIN Project 2015, prot. 2015KA3XFH).

Authors' contributions

PC: Conceptualization, Supervision, Project administration, Funding acquisition, Investigation, Validation, Writing & editing. FDC: Investigation, Formal analysis, Validation. SD: Investigation, Formal analysis, Validation. AL: Formal analysis, Data curation, Visualization. AP: Conceptualization, Supervision, Project administration, Funding acquisition, Writing & editing.

Competing interests

The authors declare that they have no competing interests.

References

1. Chen, X., Li, Z., Liu, M., Jiang, C., Che, Y., 2015. Microbial community and functional diversity associated with different aggregate fractions of a paddy soil fertilized with organic manure and/or NPK fertilizer for 20 years. J. Soils Sediments 15, 292–301. https://doi.org/10.1007/s11368-014-0981-6. 2. Fierer, N., 2017. Embracing the unknown: disentangling the complexities of the soil microbiome. Nat. Rev. Microbiol. 15, 579–590. https://doi.org/10.1038/nrmicro.2017.87. 3. Hamm, A.C., Tenuta, M., Krause, D.O., Ominski, K.H., Tkachuk, V.L., Flaten, D.N., 2016. Bacterial communities of an agricultural soil amended with solid pig and dairy manures and urea fertilizer. Appl. Soil Ecol. 103, 61–71. https://doi.org/10.1016/j.apsoil.2016.02.015 4. Looft, T., Johnson, T.A., Allen, H.K., Bayles, D.O., Alt, D.P., Stedtfeld, R.D., Sul,W.J., Stedtfeld, T.M., Chai, B., Cole, J.R., Hashsham, S.A., Tiedje, J.M., Stanton, T.B., 2012. In-feed antibiotic effects on the swine intestinal microbiome. Proc. Natl. Acad. Sci. U. S. A. 109, 1691–1696. https://doi.org/10.1073/pnas.1120238109. 5. Riber, L., Poulsen, P.H.B., Al-Soud, W.A., Skov Hansen, L.B., Bergmark, L., Brejnrod, A., Norman, A., Hansen, L.H., Magid, J., Sørensen, S.J., 2014. Exploring the immediate and long-term impact on bacterial communities in soil amended with animal and urban organic waste fertilizers using pyrosequencing and screening for horizontal transfer of antibiotic resistance. FEMS Microbiol. Ecol. 90, 206–224. https://doi.org/10.1111/1574-6941.12403. 6. Xie,W.Y., Yuan, S.T., Xu, M.G., Yang, X.P., Shen, Q.R., Zhang,W.W., Su, J.Q., Zhao, F.J., 2018. Long-term effects of manure and chemical fertilizers on soil antibiotic resistome. Soil Biol. Biochem. 122, 111–119. https://doi.org/10.1016/j.soilbio.2018.04.009. 7. Zhang, Y.J., Hu, H.W., Gou, M., Wang, J.T., Chen, D., He, J.Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environ. Pollut. 231, 1621–1632. https://doi.org/10.1016/j.envpol.2017.09.074.

IS SERBIA READY FOR THE ONE HEALTH APPROACH IN THE FIELD OF AMR CONTROL?

Ivana Ćirković[1*], Dragana D. Božić[2], Zorana Kovačević[3]

[1]*University of Belgrade, Faculty of Medicine, Institute of Microbiology and Immunology, Belgrade, Serbia*

[2]*University of Belgrade, Faculty of Pharmacy, Department of Microbiology and Immunology, Belgrade, Serbia*

[3]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: cirkoviciv@yahoo.com*

Abstract

Antimicrobial resistance (AMR) is a complex, multidimensional problem that can harm human and animal health. Hence, it is of utmost importance to include integrated and holistic multisectoral One Health approach in tackling AMR and in particular the need for better integration of aquatic ecosystem issue into current approaches. Although first Serbian National Programme with Action Plan to control AMR 2018-2021 has elements of intersectoral collaboration and coordination, water ecosystem was neglected.

In terms of the density of their occurrence and the physicochemical water quality parameters, The Republic of Serbia is among the wealthiest regions on the European continent, with almost 1,200 registered water sources. However, the development of the wastewater system is significantly behind the development of water supply system. Serbia has 44 wastewater treatment plants (WWTPs) for municipal wastewaters, with only six of them being operational.

A pilot study called "Wastewater and river waters are reservoirs of clinically relevant carbapenemase-producing *Enterobacteriaceae*" was conducted in 2017. The aim of the study was to evaluate the presence of carbapenem-resistant enterobacteria (carbapenems are last-resort antibiotics) in wastewater and river water (the Sava and the Danube) in Belgrade. This study showed the presence of carbapenem-resistant *Enterobacteriaceae* in water systems in Belgrade and highlighted the potential role of aquatic environments as reservoirs of clinically relevant antibiotic-resistant bacteria.

Therefore, we need to raise awareness of abundance and ample scope of the AMR issue in water sector in Serbia, emphasising importance of inclusion of preventive activities and mitigating measures in future AMR Action Plan.

Key words: AMR, One health approach, Action plan, water ecosystem

"One Health" is defined as "The collaborative effort of multiple disciplines — working locally, nationally, and globally — to attain optimal health for people, animals and the environment". Various emerging health issues are linked to increasing contact between humans and animals (about 1,500 microbes are known to infect humans, and 61% of them come from animals), intensification and integration of food production, and the expansion of international travel. As the number of new infectious diseases emerged in the 20th century, the scientists began to recognize the challenges that societies face regarding these threats that largely come from animals. There are many One health initiatives and one of them is One health initiative against antimicrobial resistance.

Antimicrobial Resistance (AMR) occurs when bacteria, viruses, fungi and parasites change over time and no longer respond to medicines making infections harder to treat and increasing the risk of disease spread, severe illness and death. AMR already presents a serious social and economic burden. It is estimated to be responsible for 25,000 deaths per year in the EU alone and 700,000 deaths per year globally. Inaction is projected to cause millions of deaths globally: it has been estimated that AMR might cause more deaths than cancer by 2050. Apart from the human suffering caused by that development, AMR also pushes up the cost of treatment and diminishes productivity due to illness. In the EU alone, it is estimated that AMR costs EUR 1.5 billion annually in healthcare costs and productivity losses. The World Bank has warned that, by 2050, drug-resistant infections could cause global economic damage on a par with the 2008 financial crisis.

AMR occurs naturally over time, usually through genetic changes. Antimicrobial resistant organisms are found in people, animals, food, plants and the environment (in water, soil and air). They can spread from person to person or between people and animals, including from food of animal origin. The main drivers of AMR include the misuse and overuse of antimicrobials; lack of access to clean water, etc. Additionally, the development and spread of AMR in the environment is also a growing concern, requiring further research. A number of scientific studies have identified the potential negative impacts of resistant microorganisms or antimicrobials on the environment.

We are currently facing with rapid global spread of multi- and pan-resistant microbes that cause infections that are not treatable with existing antimicrobial medicines.

In recent times, the WHO has identified 12 bacterial species and their accompanying AMR profiles as the most considerable threat to public health. These AMR bacteria have been divided into in three priority classes, e.g. carbapenem-

resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacterales* (priority "critical") or vancomycin-resistant *Enterococcus* spp. and methicillin-resistant *Staphylococcus aureus* (priority "high") based on their impact on human health and the urgency for developing new antibiotics to treat resistant infections.

Therefore, AMR is a complex problem that requires a united multisectoral approach. The One Health approach brings together multiple sectors and stakeholders engaged in human, animal and plant health, food and feed production and the environment to communicate and work together in the design and implementation of programmes, policies, legislation and research to attain better public health outcomes. Recent national and multinational strategies to address the urgency of AMR include the United States National Action Plan for combating antibiotic-resistant bacteria, World Health Organization Global Action Plan on Antimicrobial Resistance, Declaration from the 2016 high-level meeting on antimicrobial resistance at the United Nations General Assembly, and the FAO/OIE/WHO Tripartite Collaboration—all of which stress the importance of a multisectoral One Health response.

Numerous studies have underlined the role of aquatic ecosystems as a major environmental reservoir of AMR, as it represents an ideal setting for antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) to persist. Antibiotics are not always completely metabolised by the host (human and animals) and can be excreted via urine and faeces into wastewater systems. Despite the fact that concentrations of antibiotic residues (AR) in aquatic ecosystems are significantly lower than antibiotic therapeutic doses, the microbial ecology of aquatic ecosystems remains affected and selection for ARB occurs.

Clinical wastewater, such as wastewater from healthcare institutions and nursing homes, are hotspots for AR, ARB and ARG. However, more than 80% of the total antibiotic consumption in the human sector is prescribed in the community in Europe and therefore these AMR indicators are also present in municipal wastewater. Livestock may also be carriers of clinically relevant ARB and ARG, as numerous antibiotics are used both in veterinary and human medicine. They are discharged into the environment by wastewater from fattening farms and slaughterhouses, thereby endangering public health.

A pilot study called "Wastewater and river waters are reservoirs of clinically relevant carbapenemase-producing *Enterobacteriaceae*" was conducted in 2017. The aim of the study was to evaluate the presence of carbapenem-resistant enterobacteria (carbapenems are last-resort antibiotics) in wastewater and river water (the Sava and the Danube) in Belgrade. This study showed the presence of carbapenem-resistant

Enterobacteriaceae in water systems in Belgrade and highlighted the potential role of aquatic environments as reservoirs of clinically relevant antibiotic-resistant bacteria.

Therefore, we need to raise awareness of abundance and ample scope of the AMR issue in water sector in Serbia, emphasising importance of inclusion of preventive activities and mitigating measures in future AMR Action Plan.

Reference

1. Aarestrup F.M., Wegener H.C., Collignon P. 2008. Resistance in bacteria of the food chain: epidemiology and control strategies. Expert Rev Anti Infect Ther, 6:733–750. http://dx.doi.org/10.1586/14787210.6.5.733. 2. Centers for Disease Control (CDC). 2013. Antibiotic Resistance Threats in the United States. Centers for Disease Control and Prevention, Atlanta, GA. 3. Collignon P. 2013. The importance of a One Health approach to preventing the development and spread of antibiotic resistance. In Mackenzie J.S., Jeggo M., Daszak P., Richt J.A. (ed), One Health: the Human-Animal-Environment Interfaces in Emerging Infectious Diseases. Springer, Berlin, Germany, 19-36. 4. Hiller C.X., Hübner U., Fajnorova S., Schwartz T., Drewes J.E. 2019. Antibiotic microbial resistance (AMR) removal efficiencies by conventional and advanced wastewater treatment processes: A review. Sci Total Environ, 685:596-608. doi: 10.1016/j.scitotenv.2019.05.315. 5. Holmes A.H., Moore L.S.P., Sundsfjord A., Steinbakk M., Regmi S., Karkey A., Guerin P.J., Piddock L.J. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet, 387:176-187 http://dx.doi.org /10.1016/S0140-6736(15)00473-0. 6. http://opendata.waterjpi.eu/dataset/stopping-antibiotic-resistance-evolution 7. https://www.norman-network.net/?q=node/106 8. https://www.un.org/pga/71/event-latest/high-level-meeting-on-antimicrobial-resistance/ 9. Laxminarayan R., Duse A., Wattal C., Zaidi A.K.M., Wertheim H.F.L., Sumpradit N., Vlieghe E., Hara G.L., Gould I.M., Goossens H., Greko C., So A.D., Bigdeli M., Tomson G., Woodhouse W., Ombaka E., Peralta A.Q., Qamar F.N., Mir F., Kariuki S., Bhutta Z.A., Coates A., Bergstrom R., Wright G.D., Brown E.D., Cars O. 2013. Antibiotic resistance: the need for global solutions. Lancet Infect Dis, 13:1057-1098 http://dx.doi.org/10.1016 /S1473-3099(13)70318-9. 10. O'Neill J. 2016. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. https://amr-review.org/. 11. One Health Commission. 2018. What is One Health? https://www .onehealthcommission.org/en/why_one_health/what_is_one_health/. Accessed January 3, 2017. 12. Robinson T.P., Bu D.P., Carrique-Mas J., Fèvre E.M., Gilbert M., Grace D., Hay S.I., Jiwakanon J., Kakkar M., Kariuki S., Laxminarayan R., Lubroth J., Magnusson U., Thi Ngoc P., Van Boeckel T.P., Woolhouse M.E.J. 2016. Antibiotic resistance is the quintessential One Health issue. Trans R Soc Trop Med Hyg, 110:377-380 http://dx.doi.org/10.1093/trstmh/trw048. 13. Savin M., Bierbaum G., Hammerl J.A., Heinemann C., Parcina M., Sib E., Voigt A., Kreyenschmidt J. 2020. ESKAPE Bacteria and Extended-Spectrum-β-Lactamase-Producing Escherichia coli Isolated from Wastewater and Process Water from German Poultry Slaughterhouses. Appl Environ Microbiol, 86(8):e02748-19. doi: 10.1128/AEM.02748-19. 14. So A.D., Shah T.A., Roach S., Ling Chee Y., Nachman K.E. 2015. An integrated systems approach is needed to ensure the sustainability of antibiotic effectiveness for both humans and animals. J Law Med Ethics 43(Suppl 3):38-45 http://dx.doi.org/10.1111/jlme.12273. 15. Torren-Edo J., Grave K., Mackay D. 2015. "One Health": the regu- lation and consumption of antimicrobials for animal use in the EU. IHAJ, 2:14–16.www.who.int 16. Waseem H, Williams MR, Stedtfeld RD, Hashsham SA. 2017. Antimicrobial Resistance in the Environment. Water Environ Res, 89(10):921-941. doi: 10.2175/106143017X15023776270179. 17. Woolhouse M.E.J., Gowtage-Sequeria S. 2005. Host range and emerging and reemerging pathogens. Emerg Infect Dis, 11:1842-1847 http://dx.doi.org/10.3201/eid1112.050997. 18. World Health Organization (WHO). 2014. Antimicrobial Resistance:Global Report on Surveillance. WHO, Geneva, Switzerland. 19. World Health Organization (WHO). 2015. Global Action Plan on Antimicrobial Resistance. WHO, Geneva, Switzerland. 20. Zinsstag J., Meisser A., Schelling E., Bonfoh B., Tanner M. 2012. From 'two medicines' to 'One Health' and beyond. Onderstepoort J Vet Res, 79:492 http://dx.doi.org/10.4102/ojvr.v79i2.492.

GUIDELINES FOR THE RATIONAL USE OF ANTIBIOTICS IN DOGS AND CATS

Fergus Allerton[1*]

[1]*Willows Veterinary Centre & Referral Service, Solihull, United Kingdom*

[*]*e-mail: fergus.allerton@veterinarycentre.com.uk*

Penicillin was discovered in 1928, first used therapeutically in 1941 and entered mass production in 1945; penicillinase was identified in 1940. While antibiotics have saved millions of lives over the last 90 years, the threat from resistant pathogens has grown and is now considered a potentially catastrophic threat to mankind. The 2016 Review on Antimicrobial Resistance (AMR), predicted that by 2050 up to 10 million people could die per year due to drug-resistant infections (a figure that exceeds the current annual global mortality due to cancer). Establishing public awareness of the threat from AMR is a vital component of a one health approach to address this challenge.

A key tenet of antibiotic use guidelines is to optimise the use of currently available antibiotics so that their potency can be maintained. Antibiotic use (whether appropriately directed or not) imposes a powerful selection pressure on bacteria and is a primary driver of AMR. Eliminating unnecessary use in people and animals will help to safeguard this invaluable resource for future generations. Antibiotic stewardship schemes encourage responsible use of antibiotics and have been shown to decrease antibiotic prescription rates and prevalence of multi-resistant infections within targeted populations.

Antibiotic stewardship: part of the toolkit to tackle AMR

Antibiotic stewardship is part of a wider one health movement that integrates measures to improve human and animal health. Globally, more antibiotics are given to healthy animals than to ill people. The majority of antibiotics used in people treat infections that they don't have. However, more people die of infectious disease (that would respond to an antibiotic) than die from a multi-resistant infection. Antibiotic stewardship should not be perceived as a restrictive entity or anti-antibiotic but about optimising antibiotic use to achieve a favourable outcome for the patient with minimal adverse effects on either the patient or the wider society. The European Network for Optimization of Veterinary Antimicrobial Treatment (ENOVAT) is a European Cooperation in Science and Technology (COST) Action – a network dedicated to

scientific collaboration with special emphasis on the development of antibiotic use guidelines and refinement of microbiological diagnostic procedures.

What resources are available to guide antibiotic selection?

Several initiatives have been prepared as part of the small animal stewardship toolkit. These include CEVA's GRAM book, and the Federation of Companion Animal Veterinary Association (FECAVA)'s recommendations. A recent publication (Allerton et al., 2021) reviewed the antibiotic use guidelines available across Europe. In the UK a project called PROTECT ME has been developed by SAMSoc and BSAVA and is freely available online. This poster includes revised guidelines and integrated QR codes that enable the document to be updated and encourage prescriber feedback. A non-prescription pad was also produced. Animal surveillance network data indicates that in the UK, antibiotics are prescribed in 50% of cases of acute diarrhoea, 40% of cases with acute respiratory signs and 20% of cases with acute vomiting (Singleton et al., 2017). It is unlikely that antibiotic therapy is necessary in more than a tiny minority of these cases. When questioned, vets often cite client pressure as the reason for antibiotic prescription while conversely, owners attribute the decision directly to the vet (Smith et al., 2018). The non-prescription form is designed to facilitate the closure of the consultation by providing an explanation for the withholding of antibiotics in cases where they are not deemed necessary.

Key recommendations featured consistently across the majority of identified antibiotic use guidelines from Europe, indicating adherence to a responsible agenda within Europe, despite national differences. Eliminating inappropriate antibiotic use for common, benign and self-limiting, indications seems a logical and achievable target; various 'do not use' recommendations appeared in all of the guidance documents. The majority also outlined appropriate non-antimicrobial therapies - a means to avoid veterinarian frustration and to provide suitable options that may address prescription pressure from the owner.

All of the guidelines encourage pathogen identification via cytology and/or bacterial culture to guide treatment decisions. Although bacterial culture with antimicrobial susceptibility testing is considered an essential stewardship tool, studies suggest that it is infrequently used in small animal practice in Europe often due to cost constraints. This is disappointing but demonstrates the difficulty of converting a key message into implemented actions. Culture and antibiotic susceptibility testing facilitates optimal drug selection and de-escalation of unnecessarily broad spectrum empiric antimicrobial therapy. What veterinarians really need though is patient-side tests that can provide this information accurately and rapidly as this would offer

greater opportunity to identify putative pathogens and avoid inappropriate empiric therapy.

The preferential use of narrow spectrum antimicrobials was another commonly (13/15) included recommendation. Despite recognition of the greater potential to contribute to AMR, the use of broad spectrum formulations, such as amoxicillin-clavulanate, far outweighs narrow spectrum alternatives in Europe. Here, external factors (e.g. drug familiarity, practice purchasing policy) are likely stronger influences in drug selection than guideline recommendations.

Prophylactic antibiotic therapy for surgery

Surgical antibiotic prophylaxis accounts for the most common indication for antibiotic use in many human hospitals and is the most frequent instance of prophylactic use in veterinary practice. This includes procedural prophylaxis (before or during surgery) and post-procedural prophylaxis. Importantly, the latter is rarely indicated (besides for known infection or cases involving severe compromise to asepsis) and yet is commonly prescribed. Meta-analyses have found procedural antibiotic prophylaxis to be an effective intervention at preventing surgical site infections in people largely independent of the type of surgery being considered. However, prophylactic administration should be reserved for cases where the risk of infection is considered high and the consequences of infection severe.

Inevitably, all surgical procedures carry a risk of infection; the benefit of antibiotic prophylaxis must be balanced against the potential risks of their use, including adverse reactions and potentiation of AMR. In Australia the Antimicrobial Prescribing Survey found that procedural and post-procedural prophylaxis were prescribed but not indicated in 10% and 40% of surgical procedures respectively (Ierano et al., 2017).

Prophylaxis is not indicated for clean procedures as defined by international guidelines. For longer (> 90 minutes), complex procedures or where the consequences of infection would be catastrophic (CNS surgery) prophylactic antibacterial use is warranted. European guidelines (including soon to be published documents from ENOVAT) encourage clinicians to always treat effectively when administering antibiotics (ensuring that appropriate concentrations can be achieved at the surgical site) by using the appropriate dose of an agent that is capable of penetrating the target site and that offers a spectrum of activity that reflects the anticipated infectious pathogens. Treatment should be re-administered during surgery if the procedure continued for more than two half-lives of the drug.

Evidence to continue antibiotic therapy beyond the end of the procedure is

lacking. It has been shown in people that an increased duration of antimicrobial prophylaxis was associated with higher odds of AKI and *Clostridioides difficile* infection in a duration-dependent fashion and, significantly, extended duration did not lead to additional surgical site infection (SSI) reduction (Branch-Elliman et al., 2019).

In most cases the appropriate antibiotics to use peri-operatively, would be an intravenous preparation of a first-generation cephalosporin or amoxicillin clavulanate administered at induction and repeated at 90 minute intervals during the procedure. Caution should be exercised when using amoxicillin clavulanate intravenously in dogs as adverse reactions (urticaria +/- hypotension) have been reported in a significant proportion of dogs given intravenous amoxicillin clavulanate during anaesthesia (Gosling and Martinez-Taboada, 2018). Consideration of the adverse effect profile is an important step in antibiotic selection and will vary by patient.

Targeting the message

A recent ethnographic study compared prescription habits of acute medical and acute surgical teams in a UK hospital (human). Striking differences in approach were identified where the surgeons' fear of negative patient outcomes overrides the fear of the unintended consequences of inappropriate antibiotic use (Charani et al., 2019). Interestingly, there is a suggestion that to achieve better adherence to a stewardship program, different language and metrics (e.g. highlighting a shorter time to discharge) may be required. These factors have not yet been investigated in veterinary medicine but it is likely that optimisation of antibiotic use across all specialties will necessitate interventions that are designed with these unique challenges in mind.

References

1. Allerton F, Prior C, Bagcigil AF, Broens E, Callens B, Damborg P, Dewulf J, Filippitzi ME, Carmo LP, Gómez-Raja J, Harpaz E, Mateus A, Nolff M, Phythian CJ, Timofte D, Zendri F, Jessen LR. Overview and Evaluation of Existing Guidelines for Rational Antimicrobial Use in Small-Animal Veterinary Practice in Europe. Antibiotics (Basel). 2021 Apr 9;10(4):409 2. Branch-Elliman W, O'Brien W, Strymish J, Itani K, Wyatt C, Gupta K. (2019) Association of Duration and Type of Surgical Prophylaxis With Antimicrobial-Associated Adverse Events. JAMA Surg. 154(7):590–598 3. British Small Animal Veterinary Association. PROTECT ME. Practice guidelines on responsible antibiotic use. https://www.bsava.com/Resources/Veterinary-resources/PROTECT-ME 4. E Charani, R Ahmad, T M Rawson, E Castro-Sanchèz, C Tarrant, A H Holmes, (2019). The Differences in Antibiotic Decision-making Between Acute Surgical and Acute Medical Teams: An Ethnographic Study of Culture and Team Dynamics, Clinical Infectious Diseases 69, 1: 12–20 5. Gosling, M.J. and F. Martinez-Taboada, Adverse reactions to two intravenous antibiotics (Augmentin and Zinacef) used for surgical prophylaxis in dogs. Vet Rec, 2018. 182(3): p. 80 6. Ierano C, Nankervis JAM, James R, Rajkhowa A, Peel T, Thursky K. Surgical antimicrobial prophylaxis. Aust Prescr. 2017;40(6):225–229 7. Singleton DA, Sánchez-Vizcaínoa F, Dawon S, Jones PH, Noble PJM, Pincbeck GL, Williams NJ and Radford AD. (2017) Patterns of antimicrobial agent prescription in a sentinel population of canine and feline veterinary practices in the United Kingdom. The Veterinary Journal 224:18–24 8. Smith, M., King, C., Davis, M., Dickson, A., Park, J., Smith, F. and Flowers, P. (2018). Pet owner and vet interactions: exploring the drivers of AMR. Antimicrobial Resistance & Infection Control, 7(1).

ANTIMICROBIAL POTENTIAL OF MEDICAL PLANTS IMPORTANT FOR USE IN VETERINARY PRACTICE

Biljana Božin[1*], Dragana Tomanić[2], Nebojša Kladar[1]

[1]*University of Novi Sad, Faculty of Medicine, Department of Pharmacy, Novi Sad, Serbia*

[2]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary medicine, Novi Sad, Serbia*

[*]*e-mail: BILJANA.BOZIN@mf.uns.ac.rs*

Abstract

The emergence of antibiotic-resistant bacteria has become a major global concern, with significant clinical and economic impact. Concerns over the increasing emergence of antibiotic-resistant bacteria due to the misuse and overuse of antibiotics and lack of new antibiotics indicate the need for alternative agents to antibiotics, such as natural compounds of plant origin. Plant extracts have shown extremely promising results for prevention, treatment, or to enhance animal health and production. Essential oils are regarded as possible substitutions of antibiotics. It should be emphasized that even though plant derivatives are defined as promising sources of new antimicrobial agents, additional studies should be conducted in order to define phytotherapy as an integral part of sustainable treatment in veterinary medicine.

Key words: animals, antimicrobial resistance, essential oils, plants, phytotherapy

Sažetak

Pojava bakterija rezistentnih na antibiotike je globalni zdravstveni problem, sa značajnim kliničkim i ekonomskim uticajem. Porast broja patogena rezistentnih na antibiotike usled zloupotrebe i prekomerne upotrebe antibiotika kao i nedostatak novih antibiotika ukazuje na potrebu za razvojem alternativa antibioticima, kao što su biljni preparati. Fitoterapija je pokazala izuzetno obećavajuće rezultate u prevenciji, lečenju kao i poboljšanju zdravlja i produktivnosti životinja. Etarska ulja se smatraju mogućom zamenom za antibiotike. Treba naglasiti da iako su lekovite biljke označene kao izvor novih antimikrobnih agenasa potrebno je sprovesti dodatna ispitivanja kako bi se fitoterapija definisala kao deo samostalne ili potporne terapije antibioticima u veterinarskoj praksi.

Ključne reči: antimikrobna rezistencija, biljke, etarska ulja, fitoterapija, životinje

Introduction

Antibiotics have impacted on animal welfare and the efficiency of animal production over the last 60 years (Callaway et al., 2021; Tamminen et al., 2018). However, these benefits have come with major consequences, including rapid development of antibiotic resistance (Callaway et al., 2021). The emergence and spread of antibiotic resistance have created a growing global threat. Both antimicrobial resistance and a possible future limitations on antibiotic use for animal agriculture could have an impact on global food security and safety, as well as on animal health and welfare (Tamminen et al., 2018). Considering the high number of veterinary antimicrobials used each year to treat livestock in EU and the possibility of cross-resistance between human and animal pathogens, alternatives to treat veterinary infectious are urgently needed (Cheng et al., 2014; Mayer et al., 2014). In line with those mentioned above, global interest in limiting antibiotics use in veterinary medicine has led to investigation and development of novel, alternative approaches in treatment and therapy of animals.

Alternative products play a crucial role in allowing farmers and veterinarians to reduce the use of antibiotics. Phytotherapy is among the most promising and widely used alternative agents based on the use of herbal remedies for therapy and prophylactic measures (Nehme et al., 2021). Phytopreparations used as therapeutics include herbal drugs, various types of herbal drug-based preparations (i.e. extracts), as well as different classes of secondary metabolites of herbal origin - alkaloids, flavonoids, saponins, essential oils (EO), etc. (Gupta et al., 2020; Russo et al., 2009)

Phytochemicals are plant-derived compounds, such as essential oils or tannins that have many proven health benefits. Phytochemicals have diverse applications, such as improving nutrient conversion, reducing food spoilage, antimicrobial activity, improving palatability, and enhancing gut health, making them an ideal category of antibiotic alternatives in the ruminant, swine, and poultry industries (Callaway et al., 2021). Very few products of herbal origin are currently registered for the treatment of livestock. Despite some medicinal plants seem to have promising potential activity in veterinary phytotherapy, clinical data are lacking, and many applications have been adapted from human evidenced phytotherapy (Calzetta et al., 2020; Mayer et al., 2014).

Medicinal plants have been extensively used worldwide in treatment and prevention of different diseases and infections in domestic animals, predominantly in livestock (Calzetta et al., 2020) due to their therapeutic efficacy, low risk of adverse events, reduced microbiological and parasitic resistance, decreased residues in animal products and environment. Additionally, medical plants are administered to a minor

extent in comparison to antibiotics or antiparasitics (Abo-El-Sooud, 2018; Blanco-Penedo et al., 2018). Active principles extracted from plants are utilized in veterinary medicine, mainly as antibacterial, antimycotic, antiparasitic, disinfectants and immunostimulants (Russo et al., 2009). Also, phytotherapy is also often utilized to support traditional treatment with synthetic drugs (Russo et al., 2009). The effective use of phytotherapy in livestock treatment is promoted by EU Regulation (Blanco-Penedo et al., 2018).

The EO of *Ocimum basilicum*, Lamiaceae has been reported as an expectorant in animals. The combination of essential oils of *Ocimum micranthum*, Lamiaceae and *Chenopodium ambrosioides*, Chenopodiaceae is claimed to treat stomach ache and colic in animals (Baser and Franz, 2010). Yang et al. (2012) tested the therapeutic effectiveness of *Angelica dahurica*, Apiaceae and *Rheum officinale*, Polygonaceae extracts in bovine mastitis therapy and they pointed out satisfactory antibacterial activity and shorter treatment duration. Hase et al. (2013) reported therapeutic efficacy of herbal spray and gel without observed side effects in treatment of bovine mastitis.

Essential oils

Essential oils are known as volatile constituents of aromatic plants, complex mixtures of terpenoid and/or nonterpenoid compounds (Baser and Franz, 2010). These products are employed in phytotherapy for a long time due to diverse properties and mostly being considered as GRAS (generally recognized as safe) (Ebani and Mancianti, 2020; Horky et al., 2019). In general, their use is eco-friendly, mostly non-toxic and consistent with nature (Horky et al., 2019). EOs are used mainly in health, cosmetic, agriculture and food industries (Baser and Franz, 2010). EOs have also been used in veterinary medicine because of their biological properties, such as antimicrobial, antifungal, antiviral, antiparasitic, expectorant, mucolytic, antipyretic, and anti-inflammatory effects (Vučinić et al., 2011; Horky et al., 2019). There are many advantages of using natural products as the antimicrobial compounds, such as fewer adverse effects, better patient tolerance, lower side effects and low resistance after prolonged exposure, wide acceptance due to their traditional applications, renewability and better biodegradability (Lopes et al., 2020; Yap et al., 2014). EOs used in veterinary medicine may be classified as follows:

1. Oils attracting animals
2. Oils repelling animals
3. Insecticidal, pest repellent, and antiparasitic oils
4. Oils used in animal feed
5. Oils used in treating diseases in animals (Baser and Franz, 2010).

EOs were shown to be active against bacterial strains of animal origin in *in vitro* and *in vivo* studies. Kovacevic et al. (2021) investigated the effectiveness of EOs of common and wild thyme against mastitis-associated pathogens in Serbia, and reported that they can be used in the development of pharmaceutical formulation as an alternative to conventional mastitis therapy. The antibacterial activity has been attributed to the presence of oxygenated monoterpenes, especially thymol and carvacrol. Furthermore, although many aromatic plants and EOs are tested, especially for antimicrobial activity, plants belonging to the genus *Thymus* (Lamiaceae) are of special interest regarding the presence of notable amounts of thymol and carvacrol, being strong antimicrobial agents (Bozin et al., 2006). EOs of the genus *Thymus* exhibit different biological properties such as antioxidant, antibacterial, antifungal, antiviral, antiparasitic, cytotoxic, carminative and spasmolytic (Fratini et al., 2021).

EOs from *Satureja* species (Lamiaceae) have demonstrated antibacterial, antiviral, antiparasitic, antioxidant, anti-inflammatory, hepatoprotective, antidiabetic and anticholesterolemic activities (Jafari et al., 2016). The antimicrobial activity of the *Satureja montana* and *Origanum vulgare* EO was evaluated against pathogens isolated in bovine mastitis cases by Kovacevic et al. (2022). Study showed promising antimicrobial activity of mentioned EOs which could be considered as one of the treatment approaches in mastitis-affected cows. The antibacterial activity has been attributed to the presence of oxygenated monoterpenes, especially thymol and carvacrol whose antibacterial activity was also confirmed by Fratini et al. (2021) and Gavaric et al. (2014).

In the last years, some veterinarians have introduced EOs in the therapy of various clinical conditions, including bacterial infections. The use of EOs in therapy of companion and farm animals should follow careful studies on the toxicity of these natural products in relation to animal species and route of administration (Ebani and Mancianti, 2020).

The antibacterial activity

EOs have been recognized for their antimicrobial properties for many years, but the exact antimicrobial mechanism is poorly understood (Cheng et al., 2014). Bacterial resistance and lack of new antibiotics on the drug market have encouraged research of antibacterial activity of EOs. Some authors reported that the individual oil components (mainly with phenolic structures) are able to exhibit a wide spectrum of antibacterial activity and that the chemical structures greatly affect the components effectiveness and their mode of antibacterial action (Dorman and Deans, 2000). Considering that a vast range of different groups of chemical compounds are present

in one EO, it is considered that antibacterial activities cannot be attributed to one specific mechanism or component (Yap et al., 2014)

Antimicrobial activity is attributed to various secondary plant metabolites, among them geranyl acetate, eugenol acetate, *trans*-cinnamaldehyde, menthol, carvacrol, thymol, geraniol, eugenol, *p*-cymene, limonene, terpinene (Burt, 2004). The greatest activity occurs when there is a high proportion of phenols and aldehydes, while weak or no activity from esters, ketones, or terpene hydrocarbons (Bassolé and Juliani, 2012). The plant extracts and EOs act on the bacterial cell by disrupting the bacterial membrane, promoting cytoplasmic membrane damage, affecting their metabolic processes or producing reactive oxygen species (Horky et al., 2019; Burt, 2004). Furthermore, it affects many cellular activities including energy production and membrane transport (Ebani and Mancianti, 2020). EOs' high content of phenolic derivatives, such as carvacrol and thymol, target the bacterial membrane transport system, causing disruption at the cytoplasmic homeostasis, affecting cell respiration and the enzyme system (Baser and Franz, 2010). The lipophilicity of the components plays an important role in the penetration of the lipid layer of the bacterial cell membrane, leading to loss of integrity and structural organization (Lopes et al., 2020). These activities are also dependent on the characteristics of the tested microorganisms (Chouhan et al., 2017). For example, gram-positive bacteria are more susceptible to EOs than gram-negative, possibly due to the difference in cell wall structure that limits the diffusion of hydrophobic compounds through the lipopolysaccharide envelope (Zhang et al., 2016). In fact, differences have been observed in relation to species, but also, within a same pathogen species, in relation to the strain (Ebani and Mancianti, 2020).

Essential oils activity against biofilm formation

Biofilm-forming microorganisms represent a serious medical problem, as they protect themselves from antibiotics and the hosts' immune response (El-Tarabily et al., 2021). These bacterial communities are adapted to adverse environmental conditions such as the presence of antimicrobials (Galovicova et al., 2021). The formation of biofilms causes failure of an antimicrobial agents, and 65–80% of infections may occur due to the formation of biofilms**.** *T. serpyllum* EO also has an inhibitory effect against biofilm-forming microorganisms (Galovicova et al., 2021). Oh et al. (2017) reported interesting anti-biofilm effect of EOs on *E. coli* and *Salmonella* strains isolated from pig feces. Inhibition of the biofilm growth has been observed with *S. typhimurium* demonstrated by EOs of garlic and thyme (Morshdy et al., 2022).

Conclusion

This review confirms potential role of medicinal plants used as an alternative in veterinary medicine. Rapid development of antibiotic resistance urges the need of finding new agents in therapy in prevention. Investigation of the antibacterial properties of EOs is of increasing interest, because therapies with alternative drugs are welcome to combat infections caused by antibiotic-resistant strains. EOs are recognized as one of the most promising approaches for the replacement of conventional antibiotics. As a result of the global increase in the demand for antibiotics, EOs are continually being tested for their antimicrobial effects.

References

1. Abo-EL-Sooud K. 2018. Ethnoveterinary perspectives and promising future. International Journal of Veterinary Science and Medicine, 6: 1-7. 2. Baser K.H.C., Franz C. 2010. Essential Oils Used in Veterinary Medicine. In: Baser KHC, Buchbauer G, editors. Handbook of Essential Oils Science, Technology and Applications. Boca Raton, London, New York: CRC Press, Taylor & Francis Group, 881-94. 3. Bassole I.H., Juliani H.R. 2012 Essential oils in combination and their antimicrobial properties. Molecules, 17: 3989-4006. 4. Blanco-Penedo I., Fernández González C., Tamminen L.M., Sundrum A., Emanuelson U. 2018. Priorities and Future actions for an effective Use of Phytotherapy in livestock—Outputs from an expert Workshop. Frontiers in Veterinary Science, 4: 248. 5. Bozin B., Mimica-Dukic N., Simin N., Anackov G. 2006. Characterization of the Volatile Composition of Essential Oils of Some Lamiaceae Spices and the Antimicrobial and Antioxidant Activities of the Entire Oils. Journal of Agricultural and Food Chemistry, 54: 1822–1828. 6. Burt S. 2004. Essential oils: Their antibacterial properties and potential applications in foods-a review. International Journal of Food Microbiology, 94: 223–253. 7. Callaway T.R., Lillehoj H., Chuanchuen R., Gay C.G. 2021. Alternatives to antibiotics: A symposium on the challenges and solutions for animal health and production. 8. Calzetta L., Pistocchini E., Leo A., Roncada P., Ritondo B.L., Palma E., di Cave D., Britti D. 2020. "Anthelminthic medicinal plants in veterinary ethnopharmacology: A network meta-analysis following the PRISMA-P and PROSPERO recommendations." Heliyon, 6: e03256. 9. Cheng G., Hao H., Xie S., Wang X., Dai M., Huang L., Yuan Z. 2014. Antibiotic alternatives: the substitution of antibiotics in animal husbandry? Frontiers in microbiology, 5: 217. 10. Chouhan S., Sharma K., Guleria S. 2017. Antimicrobial Activity of Some Essential Oils-Present Status and Future Perspectives. Medicines, 4: 58. 11. Dorman H.J., Deans S.G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of applied microbiology, 88: 308–16. 12. Ebani V.V., Mancianti F. 2020. Use of essential oils in veterinary medicine to combat bacterial and fungal infections. Veterinary sciences, *7*: 193. 13. El-Tarabily K.A., El-Saadony M.T., Alagawany M., Arif M., Batiha G.E., Khafaga A.F., Elwan H.A., Elnesr S.S., Abd El-Hack M.E. 2021. Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. Saudi Journal of Biological Sciences, 28: 5145-5156. 14. Evangelopoulou G., Solomakos N., Ioannidis A., Pexara A., Burriel A.R. 2019. A comparative study of the antimicrobial activity of oregano, rosemary and thyme essential oils against Salmonella spp. Biomedical Research and Clinical Practice, 4: 1-7. 15. Fratini F., Giusti M., Mancini S., Pisseri F., Najar B., Pistelli L. 2021. Evaluation of the in vitro antibacterial activity of some essential oils and their blends against Staphylococcus spp. isolated from episodes of sheep mastitis. Rendiconti Lincei. Scienze Fisiche e Naturali, 32: 407-416. 16. Galovičová L., Borotová P., Valková V., Vukovic N.L., Vukic M., Terentjeva M., Štefániková J., Ďúranová H., Kowalczewski P.L., Kačániová M. 2021. Thymus serpyllum Essential Oil and Its Biological Activity as a Modern Food Preserver. Plants, 10: 1416. 17. Gavaric N., Mozina S.S., Kladar N., Bozin B. 2015. Chemical Profile, Antioxidant and Antibacterial Activity of Thyme and Oregano Essential Oils, Thymol and Carvacrol and Their Possible Synergism. Journal of essential oil-bearing plants, 18: 1013–1021. 18. Gupta R., Kumar S. Khurana R. 2020. Essential oils and mastitis in dairy animals: A review. Haryana Veterinarian, 59: 1–9. 19. Hase P.B., Digraskar S., Ravikanth K., Dandle M., Maini S. 2013. Management of subclinical mastitis with mastilep gel and herbal spray (AV/AMS/15). International Journal of Pharmaceutics, 2: 64-67. 20. Horky P., Skalickova S., Smerkova K., Skladanka J. 2019. Essential oils as a feed additives: Pharmacokinetics and potential toxicity in monogastric animals. Animals, 9: 352. 21. Jafari F., Ghavidel F., Zarshenas M.M. 2016. A critical overview on the pharmacological and clinical aspects of popular Satureja Species. Journal of Acupuncture and Meridian Studies, 9: 118–127. 22. Lopes T.S., Fontoura P.S., Oliveira A., Rizzo F.A., Silveira S., Streck A.F. 2020. Use of plant extracts and essential oils in the control of bovine mastitis. Research in Veterinary Science, 131: 186–193. 23. Mayer M., Vogl C.R., Amorena M., Hamburger M., Walkenhorst M. 2014. Treatment of organic livestock with medicinal plants: a systematic review of European ethnoveterinary research. Complementary Medicine Research, 21: 375-386. 24. Morshdy A.E.M., El-Tahlawy A.S., Qari S.H., Qumsani A.T., Bay D.H., Sami R., Althubaiti E.H., Mansour A.M., Aljahani A.H., Hafez A.E.S.E., Mahmoud A.F.A. 2022. Anti-Biofilms' Activity of Garlic and Thyme Essential Oils against Salmonella typhimurium. Molecules, 27: 2182. 25. Nehme R., Andrés S., Pereira R.B., Ben Jemaa M., Bouhallab S., Ceciliani F., López S., Rahali F.Z., Ksouri R., Pereira D.M. Abdennebi-Najar L. 2021. Essential oils in livestock: From health to food quality. Antioxidants, 10: 330. 26. Oh S.Y., Yun W., Lee

J.H., Lee C.H., Kwak W.K., Cho J.H. 2017. Effects of essential oil (blended and single essential oils) on anti-biofilm formation of Salmonella and Escherichia coli. Journal of animal science and technology, 59: 1-5. 27. Russo R., Autore G., Severino L. 2009. Pharmaco-toxicological aspects of herbal drugs used in domestic animals. Natural product communications, 4: 1777 – 1784. 28. Tamminen L.M., Emanuelson U., Blanco-Penedo I. 2018. Systematic review of phytotherapeutic treatments for different farm animals under European conditions. Frontiers in veterinary science, 5: 140. 29. Yang W.T., Ke C.Y., Wu W.T., Lee R.P., Tseng Y.H. 2019. Effective treatment of bovine mastitis with intramammary infusion of Angelica dahurica and Rheum officinale extracts. Evidence-Based Complementary and Alternative Medicine, 7242705. 30. Yap P.S.X., Yiap B.C., Ping H.C., Lim S.H.E. 2014. Essential oils, a new horizon in combating bacterial antibiotic resistance. The open microbiology journal, 8: 6.

THE ROLE OF VETERINARIANS IN THE CONTROL OF THE ANTIMICROBIAL RESISTANCE

Zorana Kovačević[1*], Dragana Tomanić[1]

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: zorana.kovacevic@polj.edu.rs*

Abstract

Antimicrobial resistance (AMR) poses the global public health concern influencing both, humans and animals and the emergence of AMR limits the possibilities for treating infectious diseases. AMR affects both humans and animals and resistance can also spread from animals to humans through the food chain or direct contact. This complex problem requires a "One Health" approach with the cooperation of all healthcare sectors as well as agriculture, finance and consumers. Veterinarians are leaders and stewards in preserving antibiotics for animals and people trough disease prevention, prudent antimicrobial use, commitment to antimicrobial stewardship, as well as using alternatives to antimicrobials. Working with animal owners and producers, veterinarians can slow antibiotic resistance by implementing disease prevention strategies and improving the use of antibiotics while also guaranteeing high-quality medical care for animal patients.

Key words: antibiotics, antimicrobial resistance, veterinarians, antimicrobial stewardship

Sažetak

Antimikrobna rezistencija (AMR) predstavlja globalni problem javnog zdravlja koji utiče i na ljude i na životinje, a pojava AMR-a ograničava mogućnosti lečenja zaraznih bolesti. AMR utiče i na ljude i na životinje, a rezistencija se takođe može širiti sa životinja na ljude putem lanca ishrane ili direktnog kontakta. Ovaj složeni problem zahteva pristup „Jednog zdravlja" uz saradnju svih zdravstvenih sektora, kao i poljoprivrede, ekonomije i potrošača. Veterinari imaju vodeću ulogu u očuvanju efikasnosti antibiotika za životinje i ljude kroz prevenciju bolesti, opreznu upotrebu antimikrobnih sredstava, posvećenost upravljanju antibioticima, kao i korišćenje alternativa antimikrobnim sredstvima. Radeći sa vlasnicima životinja i proizvođačima, veterinari mogu usporiti razvoj rezistencije primenom strategija prevencije bolesti i racionalnom upotrebom antibiotika, istovremeno obezbeđujući visokokvalitetnu

medicinsku negu za životinje.

Ključne reči: antibiotici, antimikrobna rezistencija, veterinari, upravljanje antibioticima

Introduction

There is increasing concern globally about the enormity of the threats posed by antimicrobial resistance (AMR) to human, animal, plant and environmental health (Palma et al., 2020). Although the development of AMR occurs naturally when microorganisms replicate themselves erroneously or when resistant traits are exchanged between them, the antimicrobial use (AMU) and misuse increase the development of resistant strains (Lloyd and Page, 2018; FDA-CVM, 2012). AMU in animals can contribute to the emergence of resistant bacteria that can be transferred to humans through the food chain or direct contact. Hence, AMR affects both humans and animals (Pomba et al., 2020; Schwarz et al., 2017; EFSA and EMA, 2017)**.** In addition, this can reduce the effectiveness of antimicrobials for treating human disease (Razzaque, 2021). Moreover, the lack of new antibiotics entering the market exacerbates the problem while the emerging and steady increase in the occurrence of bacteria that are resistant to multiple antibiotics has become a global public health threat due to the lack of therapeutic options to treat certain infections in humans (Allcock et al., 2017).

To limit the development of resistance for the benefit of animal and public health, European Medicines Agency (EMA) is promoting the prudent use of antimicrobials in animals and is engaged in numerous activities to address the threat arising from the use of medically important antimicrobials in food-producing animals. Furthermore, in veterinary medicine, EMA is promoting prudent use of antimicrobials in animals, collecting data on the use of veterinary antimicrobials in the European Union (EU), and providing scientific recommendations on the use of specific antimicrobials in animals (EMA, 2019). Interestingly, Serbia still is not part of it of the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) reporting of data on the use of antimicrobial agents in animals (ESVAC, 2020). Moreover, it is established that in low income countries (such as Serbia) AMR poses a significant threat to the public health due not only to the health-care challenges these countries face, but also to an increase in small-scale intensive animal production, being more severe by poor sanitation infrastructure (Timbrook et al., 2017). Additionally, in order to combat AMR, Serbia formulated its National Antimicrobial Resistance Control Program for 2019–2023, where among other strategies, special attention is given on increasing awareness among those who prescribe antimicrobials,

including veterinarians (Timbrook et al., 2017). This program is established in 2019, following the resolution the United Nations by all countries.

Education on AMR issue among all healthcare professionals (including veterinarians) is highlighted in the World Health Organization (WHO) Global Action Plan on AMR in 2015 (WHO, 2015). Veterinarians are leaders and stewards in preserving antibiotics for animals and people. Working with animal owners and producers, veterinarians can slow AMR by implementing disease prevention strategies and improving the use of antibiotics while also guaranteeing high-quality medical care for animal patients. This could be achieved by disease prevention, prudent use of antimicrobials that relay on AMU guidelines and use of antibiograms, then commitment to antimicrobial stewardship and use of alternatives to antimicrobials.

The disease prevention

Implement best practice for animal husbandry, vaccination, nutrition, and biosecurity represents major activities in disease prevention and control (MacPhillamy et al., 2022). Intensive production of livestock animals, means a high concentration of animals in a relatively small space, which requires determination of biosecurity measures aimed at preserving the health of animals, preventing the introduction and spread of the disease and preservation of production (Stankovic and Hristov, 2009). Biosecurity messures are intended to prevent adverse situations and improve the business, which, in essence, is the prevention of disease. Biosecurity involves the removal or reduction of specific factors (effective disease control systems, such as vaccination and dehelmintization) and nonspecific factors (through the proper ways of rearing and breeding, good hygiene, reduction of exposure of pigs to all forms of stress) of disease outbreaks and spread to a minimum (Stankovic et al., 2010). Safe and nutritionally balanced feed are effective preventive measures to help animals to cope with pathogens by enhancing the overall animal health welfare status through specific feeding strategies, feed composition, feed formulations or feed processing (EFSA and EMA, 2017)

Vaccines are promising alternatives to antibiotics, recognized as environmentally friendly (Hoelzer et al., 2018; Pastoret and Jones, 2004). For this reason, vaccines would lessen the need for antibiotics to control diseases and offer veterinary practitioners much needed tools. Moreover, the growing public interest in production of food without pharmaceutical residues, supports the importance of the development of new veterinary vaccines (Sander et al., 2020). In this regard, the development of vaccines is relevant issue for human and animal health. Radinovic et al. (2021) highlighted the role of vaccination in prevention of mastitis caused by

environmental agents. The effects achieved by the vaccine are a reduction in the number of somatic cells in milk, a reduction in the total number of bacteria in milk and an increase in the amount of milk. A large number of studies indicate that a vaccine prepared from inactivated bacteria reduces the occurrence of clinical and subclinical mastitis (Magas and Vakanjac, 2012).

Vaccination is useful in the control of many diseases, if it conforms to the country's effective disease control programme. However, vaccination on its own will not usually achieve the desired results unless the vaccination programme is part of an integrated control strategy utilizing a combination of control measures (OIE, 2014).

Furthermore, in Animal Health Strategy given by the EU is highlighted that prevention is better than cure (European Commission, 2007). Educate people who engage with animals on how to prevent disease (Grönthal et al., 2018). For example, the regular and close contact between companion animals and humans provides excellent opportunities for interspecies transmission of resistant bacteria and their resistance genes in either direction (Reynolds et al., 2019). Hence, the increasing trends and prevalence of carbapenem-resistant bacteria observed in many companion animals is of major public health concern as companion animals could be reservoirs of CP bacteria, thus acting as direct players in the transmission of these resistant bacteria to humans (Pomba et al., 2017). Furthermore, it is important to educate veterinarians how carbapenem-resistant Enterobacterales in companion animals can affect veterinary practice and how to implement infection prevention practices to keep vets patients and pet owners health.

The prudent selection and use of antimicrobials

Responsible and prudent use includes implementing practical measures and recommendations intended to improve animal health and animal welfare while preventing or reducing the selection, emergence and spread of antimicrobial-resistant bacteria in animals and humans. Such measures include: 1) ensuring the rational use of antimicrobial agents in animals with the purpose of optimising both their efficacy and safety; 2) complying with the ethical obligation and economic need to keep animals in good health; 3) preventing or reducing the transfer of resistant micro-organisms or resistance determinants within animal populations, the environment and between animals and humans; 4) contributing to the maintenance of the efficacy and usefulness of antimicrobial agents used in animal and human medicine; 5) protecting consumer health by ensuring the safety of food of animal origin with respect to residues of antimicrobial agents (OIE, 2021) .

It is necessary to preserve and extend the effectiveness of known and available antimicrobial drugs with prudent AMU. This is why we should strive to minimize the potential for AMR, while maximizing the antimicrobial effect, especially with good quality antimicrobial stewardship (AMS) programs. Furthermore, one established method to combat AMR is the implementation of good practice AMU guidelines (Wangmo et al., 2021; Kovacevic et al., 2020). Interestingly, even though the use of antimicrobials as growth promotors is banned in Europe, in some countries across the world these drugs are still used for this purpose (Van Boeckel et al., 2017).

The role of veterinary specialists, such as microbiologists, pharmacologists and epidemiologists, has to be improved and better emphasized. Educational tools have to be carefully applied not only among professionals but also for consumers and citizens (Vercelli et al., 2022). Furthermore, WHO principles pointed out the importance of training veterinarians on this issue on graduate and postgraduate level, also as continuing education (WHO, 2000).

When it comes to veterinarian impact to combat AMR, specifically related to the food producing animals, antibiotic usage can be reduced without reducing productivity and profitability: prudent use, complementary strategies to increase animal welfare, hygiene practice, administration of probiotics and vaccines can significantly reduce the usage of antibiotics (Levy, 2014). In Serbia, most farm animal veterinarians realize the effect AMU and rational prescribing have on the AMR issue and are using AMU guidelines to help them in their everyday work. Although, there is dissatisfaction regarding the amount of available local guidelines and specifically the use of antibiotics by unqualified people (Vidovic et al., 2022).

Commit to antimicrobial stewardship

Antimicrobial stewardship (AMS) is central to efforts to ensure access to effective antimicrobials for all who need them, today and tomorrow. The term antimicrobial stewardship emerged relatively recently, and is being applied in an increasingly diverse range of contexts; many current definitions of AMS are technical and focus on prescriptions. Dyar et al. (2017) have suggested that AMS is a coherent set of actions which promote using antimicrobials responsibly. Actually, AMS promote using antimicrobials in ways that ensure sustainable access to effective therapy for all who need them. The specific actions vary depending on the actor, but share many commonalities at different levels within a healthcare system, as well as between human and animal health. Unfortunately, farm animal veterinarians in Serbia showed a substandard understanding of AMS, making further education and

promotion of AMS concepts one of the priorities in our fight for the reduction of AMU and AMR (Vidovic et al., 2022).

At country level, higher resistance in companion animals seems to follow trends of higher antimicrobial use (both food-producing and companion animals), as reported by ESVAC (2018), suggesting that resistance levels in companion animals might be driven by other factors than only direct selective pressure by antimicrobial treatment in cats and dogs. In the past, the use of antibiotics in livestock has been considered the only responsible for the widespread of AMR but also pets, environment, wild animals and exotic animals has a key role, often underestimated but not negligible (Vercelli et al., 2022).

Figure 1. The figure represents the five key points of the educational plans for professionals and consumers designed by World Health Organization (WHO) global action plan (adapted by Collignon and McEwen, 2019)

At this purpose, restrictive regulations have been relatively recently imposed to veterinarians in the EU countries. Nevertheless, the issue does not seem to be ameliorated, indicating an intrinsic bias while considering individual responsibilities (e.g., veterinary medicine instead of human medicine), leaving space to the holistic vision that everyone who used antibiotics has co-share contribute in the warning phenomenon of antibiotic resistance (Palma et al., 2020). Veterinarians could implement practice-level stewardship activities, including documenting antibiotic use data, examining use practices, and serving as an educational resource for clients. Furthermore, all veterinary specialists could engage veterinary diagnostic laboratories to provide antibiograms to help determine which antibiotics will effectively treat infections.

Alternatives to antimicrobials

Besides scientific research to improve knowledge and to discover novel classes of antimicrobials and appropriate alternatives are required to ensure public health. Interestingly, veterinarians in Serbia had very positive attitudes towards the possibilities of alternatives to antibiotics (Vidovic et al., 2022). In a number of alternative solutions, most often mentioned in recent literature are essential oils, probiotics and prebiotics.

Essential oils (EOs) based formulations are recognized as one of the possible alternatives due to the numerous pharmacological effects that these compounds have (Cheng et al., 2020; Yang et al., 2019). The advantages of the EOs over antibiotics, such as non-toxicity, biodegradability and reduced withdrawal period, make their research of increasing interest (McPhee et al., 2011; Basakaran et al., 2009). In addition, the EOs does not increase antibiotic resistance during long-term use, which is their main advantage as promising candidates for novel drugs in treatment of bacterial and fungal infections (Kovačević et al., 2021; Ebani and Mancianti, 2020).

The EOs have wide application in a number of industries, such as food, agricultural pharmaceuticals, cosmetics (Bakkali et al., 2008). Due to their bactericidal, fungicidal and virucidal effects, the EOs are used in the treatment of diseases caused by microorganisms in both, human and veterinary medicine (Vučinić et al., 2012). Numerous *in vitro* studies have demonstrated that EOs and their active compounds can exhibit different antimicrobial behavior in relation to the tested pathogens (Kovacevic et al., 2022; Tomanic et al., 2022; Kovacevic et al., 2021; Szweda et al., 2018; Pasca et al., 2017; Abboud, 2015). On the other hand, there is a lack of information about their effectiveness in treating infections in animals. According to the literature, the presence of phenolic components (carvacrol and thymol) is considered to be the main reason for the good antibacterial potential of these oils (Veldhuizen et al., 2006; Burt et al., 2005; Burt, 2004).

Probiotics and prebiotics are regarded as possible strategies to reduce or even eliminate routine antimicrobial use in animal production (Anadón et al., 2019). Probiotics can be used as drug alternatives in growth promoters and in the prevention or treatment of various infectious diseases (Kober et al., 2022). Probiotics are defined as live strains of strictly selected microorganisms which, when administered in adequate amounts beneficially affects the host animal by improving its intestinal balance (Silva et al., 2020; Anadón et al., 2019). The usage of probiotics is having similar effects as when using antibiotics but avoiding possible side effects (residues, withdrawal period, resistance, allergies, genotoxicity) (Sefer et al., 2015). Over the past decade, the use of probiotics as feed supplements in animal production has

increased considerably due to the ban on antibiotic growth promoters in livestock (Allen et al., 2013). Recently, the use of probiotics in livestock has been suggested to significantly improve their health, immunity, growth performance, nutritional digestibility (Markowiak and Śliżewska, 2018). Live yeast (*Saccharomyces*) formulations are by far the most commonly marketed products for ruminants (Cagle et al., 2020). Live yeasts have been proven to boost performance in dairy ruminants by improving their immunity. Additionally, it was demonstrated that the supplementation of probiotic yeast products improved the inflammatory response of cattle (Kober et al., 2022).

Prebiotics are defined as food components for live microorganisms that are considered beneficial for health and well-being. Prebiotics have been studied and used for companion animals and animal husbandry, including livestock, poultry, and aqua-culture (Anadón et al., 2019). It should be emphasized that the use of probiotics and prebiotics is safe, does not have a negative impact on the natural environment, and reduces the demand for antibiotic-based growth stimulators (Markowiak and Śliżewska, 2018).

Conclusion

The role of all healthcare professionals, as well as veterinarians in the control of AMR is huge. Implementing best practice for animal husbandry, vaccination, nutrition, and biosecurity, as well as using antimicrobials prudently, being committed to AMS and using alternatives to antimicrobials are some of possibilities to combat AMR. Furthermore, with small steps on everyday level, veterinarians could have huge impact on AMR spread and development, especially by raising awareness about this huge global problem and AMR gene transmission possibilities among farmers, pet owners and other citizens.

Acknowledgment:

This research was supported by the Science Fund of the Republic of Serbia, PROMIS, #GRANT No 6066966, InfoBomat and ENOVAT COST project CA18217

References

1. Abboud M., El Rammouz R., Jammal B., Sleiman M. 2015. In vitro and in vivo antimicrobial activity of two essential oils Thymus vulgaris and Lavandula angustifolia against bovine Staphylococcus and Streptococcus mastitis pathogen. Middle East Journal of Agriculture Research, 4: 975-983. 2. Allcock S., Young E.H., Holmes M., Gurdasani D., Dougan G., Sandhu M.S., Solomon L., Török M.E. 2017. Antimicrobial resistance in human populations: challenges and opportunities. Global health, epidemiology and genomics, 2: e4. 3. Allen H.K., Levine U.Y., Looft T., Bandrick M. Casey T.A. 2013. Treatment, promotion, commotion: antibiotic alternatives in food-producing animals. Trends in microbiology, 21: 114-119. 4. Anadón A., Ares I., Martínez-Larrañaga M.R., Martínez M.A. 2019. Prebiotics and probiotics in feed and animal health. In Nutraceuticals in Veterinary Medicine. Springer, Cham. pp. 261-285. 5. Bakkali F., Averbeck S., Averbeck D., Idaomar M. 2008. Biological effects of essential oils—A review. Food and Chemical Toxicology, 46: 446–475. 6. Baskaran S.A., Kazmer G.W., Hinckley L., Andrew S.M., Venkitanarayanan K. 2009. Antibacterial effect of plant-derived

antimicrobials on major bacterial mastitis pathogens in vitro. Journal of dairy science, 92: 1423-1429. 7. Burt S. 2004. Essential oils: Their antimicrobial properties and potential applications in foods: A review. International journal of food microbiology, 94: 223–253. 8. Burt S.A., Vlielander R., Haagsman H.P., Veldhuizen E.J. 2005. Increase in activity of essential oil components carvacrol and thymol against Escherichia coli O157: H7 by addition of food stabilizers. Journal of Food Protection, 68: 919–926. 9. Cagle C.M., Fonseca M.A., Callaway T.R., Runyan C.A., Cravey M.D., Tedeschi L.O. 2020. Evaluation of the effects of live yeast on rumen parameters and in situ digestibility of dry matter and neutral detergent fiber in beef cattle fed growing and finishing diets. Applied Animal Science, 36: 36–47. 10. Chapter 6.10. - Responsible and prudent use of antimicrobial agents in veterinary medicine OIE - Terrestrial Animal Health Code - 19/07/2021, Available online: https://www.woah.org/fileadmin/Home/eng/Health_standards/tahc/current/chapitre_antibio_use.pdf (accessed on June 6, 2022). 11. Cheng W.N., Han S.G. 2020. Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments—A review. Asian-Australasian Journal of Animal Sciences, 33: 1699-1713 12. Dyar O.J., Huttner B., Schouten J., Pulcini C. 2017. What is antimicrobial stewardship?. Clinical microbiology and infection, 23: 793-798. 13. Ebani V.V., Mancianti F. 2020. Use of Essential Oils in Veterinary Medicine to Combat Bacterial and Fungal Infections. Veterinary sciences, 7: 193. 14. European Commission. Directorate-General for Health and Consumer Protection. 2007. A New Animal Health Strategy for the European Union (2007-2013) where" Prevention is Better Than Cure″ (Vol. 539). Office for Official Publications of the European Communities. Available online: https://ec.europa.eu/food/system/files/2016-10/ah_policy_strategy_2007-13_en.pdf (accessed on June 6, 2022). 15. European Medicines Agency, ESVAC. 2018. Sales of Veterinary Antimicrobial Agents in 19 EU/EEA Countries in 2010: Second ESVAC Report. Available online: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-19-european-union/european-economic-area-countries-2010-second-european-surveillance-veterinary-antimicrobial_en.pdf (accessed on June 6, 2022). 16. European Medicines Agency, ESVAC. 2020. Sales of veterinary antimicrobial agents in 31 European countries. Trends from 2010 to 2018. Tenth ESVAC report. Available online: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2018-trends-2010-2018-tenth-esvac-report_en.pdf (accessed on June 5, 2022) 17. EMA: Antimicrobial resistance in veterinary medicine Available online: https://www.ema.europa.eu/en/veterinary-regulatory/overview/antimicrobial-resistance-veterinary-medicine (accessed on June 5, 2022) 18. EMA Committee for Medicinal Products for Veterinary Use (CVMP) and EFSA Panel on Biological Hazards (BIOHAZ), Murphy, D., Ricci, A., Auce, Z., Beechinor, J.G., Bergendahl, H., Breathnach, R., Bureš, J., Duarte Da Silva, J.P., Hederová, J. and Hekman, P., 2017. EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). EFSA Journal, 15(1), p.e04666. 19. Food and Drug Administration-Centre for Veterinary Medicine (FDA-CVM). 2012. The Judicious Use of Medically Important Antimicrobial Drugs in Food-Producing Animals. Available online : https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cvm-gfi-209-judicious-use-medically-important-antimicrobial-drugs-food-producing-animals (accessed on 6 June 2022). 20. Grönthal T., Österblad M., Eklund M., Jalava J., Nykäsenoja S., Pekkanen K., Rantala M. 2018. Sharing More than Friendship—Transmission of NDM-5 ST167 and CTX-M-9 ST69 Escherichia Coli between Dogs and Humans in a Family, Finland, 2015. Eurosurveillance, 23:1700497. 21. Hoelzer K., Bielke L., Blake D.P., Cox E., Cutting S.M., Devriendt B., Erlacher-Vindel E., Goossens E., Karaca K., Lemiere S., Metzner M. 2018. Vaccines as alternatives to antibiotics for food producing animals. Part 2: new approaches and potential solutions. Veterinary research, 49: 1-15. 22. Kober A.H., Riaz Rajoka M.S., Mehwish H.M., Villena J., Kitazawa H. 2022. Immunomodulation Potential of Probiotics: A Novel Strategy for Improving Livestock Health, Immunity, and Productivity. Microorganisms, 10: 388. 23. Kovacevic Z., Blagojevic B., Suran J., Horvat O. 2020. Mapping knowledge and comprehension of antimicrobial stewardship and biosecurity among veterinary students. PLOS ONE, 15: e0235866. 24. Kovačević Z., Radinović M., Čabarkapa I., Kladar N., Božin B. 2021. Natural agents against bovine mastitis pathogens. Antibiotics, 10: 205. 25. Kovačević Z., Kladar N., Čabarkapa I., Radinović M., Maletić M., Erdeljan M., Božin B. 2022. New perspective of Origanum vulgare L. and Satureja montana L. essential oils as bovine mastitis treatment alternatives. Antibiotics, 10: 1460. 26. Levy S. 2014. Reduced antibiotic use in livestock: how Denmark tackled resistance. Environmental Health Perspectives, 122: 160-165. 27. Lloyd D.H., Page S.W. 2018. Antimicrobial stewardship in veterinary medicine. Microbiology spectrum, 6: 6-3. 28. MacPhillamy I.B., Young J.R., Vitou S., Chanphalleap H., Sothoeun S., Windsor P.A., Toribio J.A.M., Bush R.D. 2022. Can improving animal health and biosecurity knowledge of para-veterinarians in Cambodia assist in addressing challenges in smallholder livestock farming? Transboundary and Emerging Diseases, 69: 559-569. 29. Magas V., Vakanjac S. 2012. Suzbijanje mastiitsa krava. Zbornik predavanja sa 23. Savetovanja veterinara Srbije, Zlatibor, 195-202. 30. Markowiak P., Śliżewska K. 2018. The role of probiotics, prebiotics and synbiotics in animal nutrition. Gut pathogens, 10: 1-20. 31. McPhee C.S., Anderson K.L., Yeatts J.L., Mason S.E., Barlow B.M., Baynes R.E. 2011. Hot topic: Milk and plasma disposition of thymol following intramammary administration of a phytoceutical mastitis treatment. International Journal of Dairy Science, 94: 1738-1743. 32. Palma E., Tilocca B. Roncada P. 2020. Antimicrobial resistance in veterinary medicine: An overview. International Journal of Molecular Sciences, 21: 1914. 33. Pașca C., Mărghitaș L.A, Dezmirean D.S., Bobiș O., Bonta V., Chirilă F., Matei I., Fiț N. 2017. Medicinal plants based products tested on pathogens isolated from mastitis milk. Molecules, 22: 1473. 34. Pastoret P.P., Jones P. 2004. Veterinary vaccines for animal and public health. Developments in biologicals, 119: 15-29. 35. Pomba C., Rantala M., Greko C., Baptiste K.E., Catry B., van Duijkeren E., Mateus A., Moreno M.A., Pyörälä S., Ružauskas M., Sanders P, Teale C., Threlfall E.J., Kunsagi Z., Torren-Edo J., Jukes H., Törneke K. 2017. Public Health Risk of Antimicrobial Resistance Transfer from Companion Animals. Journal of Antimicrobial Chemotherapy, 72: 957–968. 36. Pomba C., Belas A., Menezes J., Marques C. 2020. The Public Health Risk of Companion Animal to Human Transmission of Antimicrobial Resistance During Different Types of Animal Infection. In Advances in Animal Health, Medicine and Production, Springer, pp. 265-278. 37. Razzaque M.S. 2021. Implementation of antimicrobial stewardship to reduce antimicrobial drug resistance. Expert Review of Anti-infective Therapy, 19: 559-562 38. Reynolds M.E., Phan H.T.T., George S., Hubbard A.T.M., Stoesser N., Maciuca I.E., Crook D.W., Timofte D. 2019. Occurrence and Characterization of *Escherichia coli* ST410 Co-Harbouring Bla_{NDM-5}, Bla_{CMY-42} and $Bla_{TEM-190}$ in a Dog from the UK. Journal of Antimicrobial Chemotherapy, 74: 1207–1211. 39. Sander V.A., Sánchez López E.F., Mendoza Morales L., Ramos Duarte V.A., Corigliano M.G., Clemente M. 2020. Use of veterinary vaccines for livestock as a strategy to control foodborne parasitic diseases. Frontiers in Cellular and Infection Microbiology, 10: 288. 40. Schwarz S., Loeffler A., Kadlec K. 2017. Bacterial resistance to antimicrobial agents and its impact on veterinary and human medicine. Advances in Veterinary Dermatology, 8: 95-110 41. Sefer D., Marković R., Nedeljković-Trailović J., Petrujkić B., Radulović S., Grdović S. 2015. The application of biotechnology in animal nutrition. Veterinarski glasnik, 69: 127-137. 42. Silva D.R., Sardi

J.D.C.O., de Souza Pitangui N., Roque S.M., da Silva A.C.B., Rosalen P.L. 2020. Probiotics as an alternative antimicrobial therapy: Current reality and future directions. Journal of Functional Foods, 73: 104080. 43. Stanković B., Hristov S. 2009. Najčešći propusti u obezbeđenju biosigurnosti na farmama goveda i svinja. Zbornik naučnih radova Instutut Agroekomik Beograd, 15: 103-109. 44. Stanković B., Hristov S., Bojkovski T.J., Maksimović N. 2010. Health status and bio-security plans on pig farms. Biotechnology in Animal Husbandry, 26: 29-35. 45. Szweda P., Zalewska M., Pilch J., Kot B., Milewski S. 2018 Essential oils as potential antistaphylococcal agents. Acta Veterinaria-Beograd, 68: 95-107. 46. Timbrook T.T., Caffrey A.R., Ovalle A., Beganovic M., Curioso W., Gaitanis M., LaPlante K.L. 2017. Assessments of opportunities to improve antibiotic prescribing in an emergency department: a period prevalence survey. Infectious diseases and therapy, 6: 497-505. 47. Tomanić D., Božin B., Čabarkapa I., Kladar N., Radinović M., Maletić M., Kovačević Z. 2022. Chemical Composition, Antioxidant and Antibacterial Activity of Two Different Essential Oils Against Mastitis Associated Pathogens. Acta Veterinaria-Beograd, 72: 45-58. 48. Van Boeckel T.P., Glennon E.E., Chen D., Gilbert M., Robinson T.P., Grenfell B.T., Levin S.A., Bonhoeffer S., Laxminarayan R. 2017. Reducing antimicrobial use in food animals. Science, 357: 1350-1352. 49. Veldhuizen E.J., Tjeerdsma-van Bokhoven J.L., Zweijtzer C., Burt S.A., Haagsman H.P. 2006. Structural requirements for the antimicrobial activity of carvacrol. Journal of Agricultural and Food Chemistry, 54: 1874–1879. 50. Vercelli C., Gambino G., Amadori M., Re G. 2022. Implications of Veterinary Medicine in the comprehension and stewardship of antimicrobial resistance phenomenon. From the origin till nowadays. Veterinary and Animal Science, 16: 100249. 51. Vidović J., Stojanović D., Cagnardi P., Kladar N., Horvat O., Ćirković I., Bijelić K., Stojanac N., Kovačević Z. 2022. Farm Animal Veterinarians' Knowledge and Attitudes toward Antimicrobial Resistance and Antimicrobial Use in the Republic of Serbia. Antibiotics, 11:64. 52. Vučinić M., Nedeljković-Trailović J., Trailović S., Ivanović S., Milovanović M., Krnjaić D. 2012. Mogućnost primene etarskih ulja u veterinarskoj medicini i stočarstvu s posebnim osvrtom na etarsko ulje origana. Veterinarski glasnik, 66: 407–416. 53. Wangmo K., Dorji T., Pokhrel N., Dorji T., Dorji J., Tenzin T. 2021. Knowledge, attitude, and practice on antibiotic use and antibiotic resistance among the veterinarians and para-veterinarians in Bhutan. PLOS ONE, 16: e0251327. 54. World Health Organization (WHO). 2000. WHO global principles for the containment of antimicrobial resistance in animals intended for food : report of a WHO consultation with the participation of the Food and Agriculture Organization of the United Nations and the Office International des Epizooties, Geneva, Switzerland 5-9 June 2000. 55. World Organization for Animal Health (OIE). 2014. Guidelines for Animal Disease Control. Available online:
https://www.woah.org/fileadmin/Home/eng/Our_scientific_expertise/docs/pdf/A_Guidelines_for_Animal_Disease_Control_final.pdf (accessed on June 6, 2022). 56. World Health Organization (WHO). 2015. Global action plan on antimicrobial resistance, Geneva. Available online: http://www.who.int/antimicrobial-resistance/global-action-plan/en/ (accessed on December 1, 2021). 57. Yang W.T., Ke C.Y., Wu W.T., Lee R.P., Tseng Y.H. 2019. Effective treatment of bovine mastitis with intramammary infusion of Angelica dahurica and Rheum officinale extracts. Evidence-Based Complementary and Alternative Medicine, 7242705.

RATIONAL USE OF ANTIBIOTICS IN FARM ANIMALS

Miodrag Radinović[1*], Ivana Davidov[1], Jovan Stanojević[1]

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: radinovic@polj.uns.ac.rs*

Abstract

Animal production on modern commercial farms is highly dependent on the use of different drugs through almost all stages of production. Antibiotics are in the first place in terms of importance and frequency of application, since veterinary medicine is number one consumer of antibiotics. As essential medicines, antibiotics are intended to preserve the health and well-being of animals and also to achieve better production results. Rational use of antibiotics is a principle that must be followed in order to achieve their best effect, and avoid harmful consequences, especially excessive use.

Key words: antibiotics, animal production, farms, animals

Sažetak

Farmska proizvodnja na savremenim komercijalnim farmama u velikoj meri zavisi od upotrebe različitih lekova u skoro svim fazama proizvodnje. Antibiotici su na prvom mestu po značaju i učestalosti primene, sobzirom da je veterinarska medicina na prvom mestu po potrošnji antibiotika. Kao esencijalni lekovi, antibiotici su namenjeni očuvanju zdravlja i dobrobiti životinja, kao i postizanju boljih proizvodnih rezultata. Racionalna upotreba antibiotika je princip koji se mora poštovati da bi se postigao njihov najbolji efekat, a izbegle štetne posledice, posebno njihova prekomerna upotreba.

Ključne reči: antibiotici, animalna proizvodnja, farma, životinje

Introduction

Livestock production is growing fast in the agricultural sector. In the last decades, production and consumption of animal products have largely increased. In the future, this increase is expected to continue in order to meet the high demand for livestock products such as meat, milk, eggs, and fish, especially in industrialized countries (Oliveira et al., 2020). This will impose necessity of widely use of antibiotics especially in poor zoohygienic conditions. In such situations increased use of antibiotics is often applied in order to preserve the health status of the animals and

enable them to produce normaly (Witte, 2000). Then it is very difficult to avoid excessive use of antibiotics so it is up to veterinary practitioners to organize application of antibiotic therapy to achieve best results with least side effects. In recent years, there has been an increase concern about the adverse effects caused by antimicrobial residues, as these drugs can cause changes in the intestinal microbiota of animals, resulting in microbial resistance.

Antibiotics in farm animals

The introduction of antibiotics as growth promoters has occurred due to the expansion and need to increase food production, as the human population has grown exponentially in recent years, which reflected on the economic capacity of the planet, resulting in the scarcity of sufficient food resources to world population (Kirchhelle, 2018).

Since 1950s, the use of antibiotics in animal feed has become a worldwide trend, which triggered by the growing dependence on the agricultural industry to increase food production faster and more effectively, following population growth. So, more than 150 antibiotics are currently used in animal production for human consumption and 90% of these antibiotics are natural products of bacteria, fungi and semi-synthetic substances derived from modifications of natural compounds, and some of them are synthetic (von Nussbaum et al., 2006). Currently about 80% of production animals destined for human consumption receive antibiotics, either to prophylactic or therapeutic function. Furthermore, the most widely used antimicrobials in the production animals designated to food consumption are β-lactams, tetracyclines, aminoglycosides, lincosamides, macrolides, and sulfonamides. However, the use of these antibiotics in animals intended for human consumption may leave residues or metabolites in meat, milk and eggs (De Briyne et al., 2014). The residue may be from the antimicrobial itself or from its secondary metabolites. Such substances may be deposited, accumulated or stored in cells, tissues, organs or edible products of animal origin. The main purpose of the use of these drugs in animals is to prevent, control or treat animal diseases or to increase production. However, these residues can accumulate in humans over time and consumption, thereby increasing the selectivity of multi-resistant bacteria to these drugs widely used in human health (Riviere and Sundof, 2001).

The use of antibiotics, in animals intended for human consumption, had as its main objective the therapeutic and prophylactic use in the control of bacterial infections. However, there was a deviation of this function, and it has been used in sub therapeutic doses, as growth promoters, a fact that contributed to the risk of

antimicrobial resistance.

The adaptations of microorganism to antibiotics and antimicrobial resistance have increased because of the excessive and incorrect use of that class of drugs in human and animal health. The regular presence of heavy metals in animal manure further increases the abundance of antibiotic resistance in bacterial populations by co-selection. In addition, the World Health Organization (WHO) classify as being of broad therapeutic use in human medicine some of antibiotics that are used to promote growth in pigs, birds and cattle. However, long-term exposure may promote the development of genetic variations of microorganisms and the production of antibiotic resistant genes, fact that results in the evolution of resistance of pathogens and bacteria. As there is currently no development of new antibiotics, antimicrobial resistance can generate a high proliferation of "super bacteria" and in the future diminish/eliminate the chances of effective treatment of diseases from those multiresistant pathogens. In addition, those micro-organisms can be transported from foods, mainly from animal sources, to the consumer through the impulsive consumption of these agricultural products and the irresponsible use of growth promoters in the production of animal origin products.

When antibiotic therapy is inevitably several principals should be followed in order to increase its efficiency and avoid or minimize side effects (Hockenhull et al., 2017).

At first before applying therapy it is necessary to choose adequate antibiotic. This can be conducted empiric, based on previous clinical experience or based on antibiogram. Since conducting of antibiogram is time consuming, veterinary clinician is often in forced to choose antibiotic base o experience, but in every situation that allows, antibiogram should be conducted. On second place is to choose route of application. This includes two different ways, systemic or local application. Local application is much more favorable so it should be selected in every situation that allows achievement of therapeutic effects. This will enable use of smaller doses of antibiotics and avoiding side effects connected with systemic administration. Regarding systemic administration it can be conducted through several different routes where every one of those has its benefits. Next step is to determine dose of antibiotic, this is regularly done by following producers instruction but in many cases veterinary clinician can correct recommended dose in order to achieve therapy effect. Final step is to determine length of application. This is often problem in clinical practice because clinicians have habit to shorten the length of application right after clinical cure is registered. So veterinary clinicians should be encouraged to conduct therapy according to producer instructions and continue with therapy even after

animal appears clinically healthy.

Conclusion

Intensive animal production in modern farms can not be realized without use of antibiotics. But veterinary clinicians must make effort to reduce excessive use of these drugs by thoroughly planning of every therapy and by respecting results of antibiogram and producers instructions.

References

1. De Briyne N, Atkinson J, Pokludová L, Borriello SP. 2014. Antibiotics used most commonly to treat animals in Europe. Vet Rec 175(13): 325. https://doi.org/10.1136/vr.102462 2. Hockenhull J, Turner AE, Reyher KK, Barrett DC, Jones L, et al. 2017. Antimicrobial use in food-producing animals: a rapid evidence assessment of stakeholder practices and beliefs. Vet Rec 181(19): 510. https://doi.org/10.1136/vr.104304 3. Kirchhelle C. 2018. Pharming animals: a global history of antibiotics in food production (1935–2017). Palgrave Commun 4: 96. https://doi. org/10.1057/s41599-018-0152-2 4. Oliveira NA, Gonçalves BL, Lee SH, Oliveira CAF, Corassin CH. 2020. Use of Antibiotics in Animal Production and Its Impact on Human Health. J Food Chem Nanotechnol 6(1):40-47 5. Riviere JE, Sundlof SF. 2001. Chemical residues in tissues of food animals. In: Adams R (eds), Veterinary Pharmacology and Therapeutics. Ames, Iowa State University Press, USA. 6. von Nussbaum F, Brands M, Hinzen B, Weigand S, Häbich D. 2006. Antibacterial natural products in medicinal chemistry--exodus or revival? Angew Chem Int Ed Engl 45(31): 5072-5129. https://doi. org/10.1002/anie.200600350 7. Witte W. 2000. Selective pressure by antibiotic use in livestock. Int J Antimicrob Agents 16 Suppl 1: S19-S24. https://doi.org/10.1016/s0924-8579(00)00301-0

SELECTION OF ANTIBIOTIC THERAPY USING EPIDEMIOLOGICAL STUDIES IN BACTERIAL VETERINARY ISOLATES

Dragana D. Božić[1*], Ivana Ćirković[2], Zorana Kovačević[3]

[1]*University of Belgrade, Faculty of Pharmacy, Department of Microbiology and Immunology, Belgrade, Serbia*

[2]*University of Belgrade, Faculty of Medicine, Institute of Microbiology and Immunology, Belgrade, Serbia*

[3]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: dragana.bozic@pharmacy.bg.ac.rs*

Abstract

Antimicrobial resistance (AMR) is a major global threat to human and animal health due to drug inefficiency, persistent infections, and a subsequent increase in the risks of severe disease and transmission. Although AMR occurs naturally, the emergence and spread of new resistance mechanisms is greatly accelerated by the overuse, misuse, inappropriate and unregulated use of antibiotics in both human and veterinary medicine, as well as in animal agriculture/aquaculture for growth promotion.

Determination of appropriate antimicrobial therapy in veterinary medicine is a great challenge for clinicians. Although there are recommendations for antibiotic usage in animals, data on minimal inhibitory concentrations (MICs), epidemiological/microbiological cut-off values (ECOFFs) and specific clinical breakpoints (CBPs) for animal species / antimicrobial agents / disease conditions are scarce, and most of the microbiological guidance are derived from human medicine.

Development of CBPs is a three-step process that includes 1) ECOFFs determination, 2) setting of a PK/PD cut-off and 3) establishment of clinical cut-off values. As the first step, precise determination of ECOFFs (i.e., distribution of MICs of wild type microorganisms lacking acquired or mutational resistance to the particular antimicrobial agent) is essential for establishment of appropriate CBPs. Currently, European Network for Optimization of Veterinary Antimicrobial Treatment - COST Action CA218217: ENOVAT has assembled the European Strain Database with more than 26000 veterinary clinical isolates, for determination of MICs and ECOFFs of various antimicrobial drugs used in veterinary medicine in Europe, that lack MICs data. These results will contribute to the establishment of new Veterinary

Antimicrobial Susceptibility Testing guidelines.

Key words: antimicrobial resistance; epidemiological/microbiological cut-off values; clinical breakpoints

Antimicrobial resistance (AMR) is one of the most important health-threat to humans and animals worldwide. Inhabiting all ecological niches, microorganisms represent a pool of AMR genes that are interchanged through the complex network of interactions between humans, domestic animals, wildlife, plants, and the environment (Giedraitiene et al., 2011). AMR occurs naturally and is a physiological mechanism that enables bacteria survival over time and in the dynamically changing environmental conditions, however, bacteria are also capable to use their evolutionary machinery to adapt to the selective pressure of antibiotics, resulting in their reduced efficacy in therapeutic treatment of human and animal infections. Accordingly, only the multisectoral approach, like the One-Health approach concept, is able to provide a detailed insight into the AMR origin and spread (Huijbers et al., 2015). In such a comprehensive approach, the overuse, misuse, inappropriate and unregulated use of antimicrobials in the human, animal, and environmental sectors, together with the global intersectoral spreading of the resistance mechanisms are identified as the major AMR driving forces (Hay et al., 2018).

Mechanisms of antimicrobial resistance

AMR is an ancient phenomenon present from the beginning of the life on our planet, with a clear distinction between innate and acquired AMR.

Innate/ Intrinsic antimicrobial resistance is the result of a slow and long evolutionary process of adaptation to the changing environmental conditions and is a feature of both pathogenic and non-pathogenic microorganisms. Major mechanisms of the intrinsic bacterial resistance are non-permeability of the bacterial cell wall to the antimicrobials, and the absence of the target molecules in the microorganisms (e.g., *Mycoplasma* spp. resistance to beta-lactams due to the absence of the cell-wall) (Olivares at al., 2013).

Molecular mechanisms of acquired resistance are well known, and most often related to presence of mobile genetic elements enabling the homogeneous dissemination of the AMR between the ecosystems of diverse sectors, such as human medicine, veterinary medicine, and the environment (Zaman et al., 2017) (Table 1).

Table 1.

Molecular mechanisms of antimicrobial resistance	
Genetic resistance	**Phenotypic resistance**
Plasmid-mediated Transposon-mediated Integron-mediated	Biofilm Persister cells Quorum sensing Swarming adaptation

Plasmid-mediated resistance

Plasmids are a very important reservoir of AMR genes that can be spread easily over the time and across species. This form of resistance represents the greatest part of the resistance spread (Olivares at al., 2013).

Transposon- mediated resistance

Transposable elements change their position within the same DNA molecule or jump between diverse DNA molecules, including plasmids, resulting in the alteration of the genome and bacterial characteristics depending on the locus of transposable elements insertion (Zaman et al., 2017).

Similarly, Integron resistance is mediated through mobilization of integrons both by the bacterial chromosome and plasmids, and can be propagated and integrated far from their original site, conferring antimicrobial protection to a wide number of microorganisms (Cambray et al., 2010). Other less-known mobile element systems also have role in horizontal gene transfer, like Integrative and Conjugative Elements (ICEs), or vertical gene transfer like Bacterial toxin-antitoxin systems (TAS).

Phenotypic persistence is not determined by genetic mutation and the genotype of the persisting cell is the same as the "wild type" (Olivares et al., 2013). Bacterial cells are not killed by the antibiotic that is administered during therapy, they remain in a quiescent status (i.e., "persister cells") that can be reversed once the influence of antibiotic is removed, and the second exposure to the antibiotic results in the bactericidal or bacteriostatic effect (Gerdes et al., 2012). Persister cells are also present in biofilms. Biofilms are surface accumulation of microbial cells enclosed in an extracellular polymeric substance matrix. AMR of bacteria in biofilms is caused by the expression of different sets of genes and metabolic states relative to their planktonic counterparts, specific biofilm microarchitecture and extensive interchange

of genetic material responsible for various mechanisms of bacterial resistance (Hall and Mah, 2017). Other two very important features of bacteria that contribute to biofilm formation as well as to antibiotic resistance are swarming and quorum sensing.

Antimicrobial use in animals - an impact on AMR

Most of the antibiotic classes that are utilized in the treatment of infectious diseases in humans are used in the veterinary sector as well, resulting in a cumulative selective pressure and subsequent reduced efficacy of the antimicrobial-based treatments in both sectors (Aarestrup et al., 2008). The greatest influence of veterinary sector to global AMR has antibiotic usage in companion animals, aquaculture, and domestic animal husbandry. Although the Methicillin-Resistant *Staphylococcus aureus* (MRSA), methicillin-resistant staphylococci, vancomycin-resistant enterococci, carbapenemase-producing enterobacteria and Extended Spectrum Beta-Lactamase (ESBL) Gram-negative bacteria have been found in companion animals (Pomba et al., 2017), the major contributor to overall resistance is still nontherapeutic overuse and misuse of antibiotics as growth promotors in aquaculture and animal herds. Amongst the antibiotic classes listed by the World Health Organization (WHO) as critically important for human medicine, six of them (aminoglycosides, macrolides, penicillins, quinolones, sulphonamides, and tetracyclines) are widely used in both terrestrial and aquaculture husbandries (O'Neill, 2015). Besides nontherapeutic usage in animals, therapeutic use of antibiotics in veterinary medicine can also contribute to the AMR, still in a far less extent. Ideally, antibiotic treatment of infectious diseases should be prescribed after accurate pathogen identification and proper antimicrobial susceptibility test (e.g., antibiogram, AST) (Aarestrup, 2019), and only to the animal with symptoms of the disease, not to the whole herd. However, it is a common practice to prescribe antibiotics empirically, and to extend the antimicrobial treatment to the whole livestock herd, to limit the pathogen spread.

Antimicrobial Susceptibility testing in Veterinary Medicine

As previously mentioned, determination of appropriate antimicrobial drug in veterinary medicine is a great challenge for clinicians. Although there are recommendations for antibiotic use in animals, data on antimicrobial susceptibility, determination of minimal inhibitory concentrations (MICs), epidemiological/microbiological cut-off values (ECOFFs) and specific clinical breakpoints (CBPs) for animal species/drug substances/disease conditions are scarce, and most of the microbiological guidance are based or derived from human medicine.

Another issue that compromises precise antibiotic therapy, and microbiological diagnostics is the lack of the European standards on AST in veterinary microbiology. The absence of adequate animal species-specific breakpoints affects the possibility for veterinary practitioners to prescribe antibiotics rationally, because there is no reliable basis for determining whether a bacterial strain is clinically resistant or not. Euopean Committee for Antimicrobial Susceptibility Testing (EUCAST) supported by European Society of Clinical Microbiology and Infectious Diseases (ESCMID) has a great number of AST standards for human microbiology. Accordingly, the EUCAST sub-committee for Veterinary Antimicrobial Susceptibility Testing - VetCAST was formed in 2015, in order to deals with all aspects of antimicrobial susceptibility testing of bacterial pathogens of animal origin and animal bacteria with zoonotic potential. VetCAST is committed to define CBPs for antimicrobial drugs used in veterinary medicine in Europe, with a goal to become an EU scientific-based operational body of EMA/CVMP for definition/approval veterinary-specific breakpoints (EUCAST, 2022). On the other hand, Clinical and Laboratory Standards Institute (CLSI) has released the 5th edition of Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals, VAST subcommittee standard that is widely used in USA (CLSI 2018, Revised 2019). However, this standard is not free of charge, and the aim of the VetCAST is to develop the European standard that will be easily accessible to everyone, and free of charge as other EUCAST standards.

Development of CBPs is a complex process that includes 1) determination of ECOFFs – the highest MIC that defines the upper end of the wild-type MIC distribution; 2) setting of a PK/PD cut-off obtained from pre-clinical pharmacokinetic data [this PK/PD break-point is the highest possible MIC for which a given percentage of animals in the target population achieves a critical value for the selected PK/PD index (fAUC/MIC or fT > MIC) and 3) the establishment of clinical cut-off values, that is the relationship between MIC and clinical cure (when possible) (Toutain et al., 2017).

As the first step, precise determination of ECOFFs (i.e., distribution of MICs of wild type microorganisms) is essential for establishment of appropriate CBPs. A microorganism is defined as wild type for a species if it has the absence of acquired or mutational resistance to the particular antimicrobial agent. The MIC or zone diameter distribution for a collection of such organisms described as a *wild type MIC* or *wild type zone diameter distribution*. To obtain the precise antimicrobial wild type distributions of microorganisms, MIC data from multiple sources, geographical areas and time periods should be collected. ECOFFs for a given bacterial species are not

affected by sampling time, source (human, animal, and environmental) and geographical origin and they represent a strictly biological parameter. A CBPs can never be lower than the ECOFF in order to prevent misclassification of strains from the wild-type population as being resistant. ECOFFs can also be used in situations where CBPs have not yet been defined, as with some topical or local agents (e.g., intra-mammary or gastro-intestinal products) or where very different breakpoints may be appropriate for different animal breeds within a same species, preventing the selection of a single CBP (Toutain et al., 2017). The example of wild type MICs for various antimicrobial agents and species can be seen on EUCAST page for Antimicrobial wild type distributions of microorganisms, and is updated every day (MIC EUCAST http://mic.eucast.org/Eucast2/).

Currently, European Network for Optimization of Veterinary Antimicrobial Treatment - COST Action CA218217: ENOVAT has assembled the European Strain Database with more than 26000 veterinary clinical isolates from 24 countries, for determination of MICs and ECOFFs of various antimicrobial drugs used in veterinary medicine in Europe, that lack MICs data (ENOVAT Deliverables – ENOVAT; D4-strain database). These results will contribute to the establishment of new Veterinary Antimicrobial Susceptibility Testing guidelines. For this activity available microbiological standards for performing AST will be employed. If not, breakpoints may vary and interpretation may be inconsistent between laboratories, or among different regions of the same country (Papich, 2013). To avoid this risk, recommendations for performing AST, interpretation of agar disk diffusion zones of inhibition breakpoints, and dilution MIC breakpoints for AST for bacteria isolated from animals should be strictly followed.

Acknowledgments

DB, IĆ and ZK are active participants of European Network for Optimization of Veterinary Antimicrobial Treatment COST Action CA218217-ENOVAT as a members of working groups WG 1-4.

DB is grateful to dr Peter Damborg, Department of Veterinary Disease Biology, University of Copenhagen, Copenhagen, Denmark, dr Annet Heuvelink, R&D department of GD Animal Health, Deventer, the Netherlands and dr Gudrun Overesch, Institute of Veterinary Bacteriology, University of Bern, Bern, Switzerland for the opportunity to participate in WG2 activities on European Strain Database and to be a co-author of the Summary Report on the European Strain Database (ENOVAT deliverable 4).

Authors' contributions

DB, IĆ and ZK equally contributed to Conceptualization, Writing, Review & Editing of the Manuscripts draft.

Competing interests

The authors declare that they have no competing interests.

References

1. Aarestrup F.M. 2019. Other Pathogens. In Antimicrobial Resistance in Bacteria of Animal Origin; ASM Press: Washington, DC , USA. 2. Aarestrup F.M.; Wegener, H.C.; Collignon, P. 2008. Resistance in bacteria of the food chain: Epidemiology and control strategies. Expert Review of Anti-Infective Therapy, 6, 733-750. 3. Cambray G., Guerout A.M., Mazel D. 2010. Integrons. Annu. Rev. Genet. 44, 141–166. 4. Clinical and Laboratory Standards Institute (CLSI). 2018. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals. 5th ed. CLSI standard VET01 (ISBN 978-1-68440-008-9 [Print]; ISBN 978-1-68440-009-6 [Electronic]). Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087 USA, 2018. 5. Gerdes K., Maisonneuve E. 2012. Bacterial Persistence and Toxin-Antitoxin Loci. Annu. Rev. Microbiol., 66, 103–123. 6. Giedraitiene A., Vitkauskiene A., Naginiene R., Pavilonis A. 2011. Antibiotic resistance mechanisms of clinically important bacteria. Medicina, 47, 137–146. 7. Hall C.W., Mah T.F.. 2017. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. FEMS Microbiol Rev, 41:276-301. 8. Hay S.I., Rao P.C., Dolecek C. Stergachis A., Lopez A., D., Murray C. J. L. 2018. Measuring and mapping the global burden of antimicrobial resistance. BMC Med 16, 78. 9. Huijbers P.M.; Blaak H.; De Jong M.C.; Graat E.A.; Vandenbroucke-Grauls, C.M.; De Roda Husman A.M. 2015. Role of the Environment in the Transmission of Antimicrobial Resistance to Humans: A Review. Environ. Sci. Technol., 49, 11993–12004. 10. O'Neill J. 2015. The O'Neill Review on Antimicrobial Resistance: Antimicrobials in Agriculture and the Environment. Rev. Antimicrob. Resist., 1–44. 11. Olivares J., Bernardini A., Garcia-Leon G., Corona F., Sanchez M.B. , Martinez J.L. 2013. The intrinsic resistome of bacterial pathogens. Front. Microbiol., 4, 103. 12. Papich MG. 2013. Antimicrobials, susceptibility testing, and minimum inhibitory concentrations (MIC) in veterinary infection treatment. Vet Clin North Am Small Anim Pract., 43(5):1079-1089. 13. Pomba C.. Rantala M., Greko C., Baptiste K.E., Catry B., van Duijkeren E., Mateus A., Moreno M.A., Pyörälä S., Ružauskas M. et al. 2017. Public health risk of antimicrobial resistance transfer from companion animals. J. Antimicrob. Chemother., 72, 957–968. 14. The European Committee on Antimicrobial Susceptibility Testing 2022. Veterinary Susceptibility Testing. http://www.eucast.org, EUCAST: Veterinary Susceptibility Testing 15. The European Committee on Antimicrobial Susceptibility Testing 2022. MIC distributions Ceftobiprole MSSA and MRSA MIC EUCAST 16. Toutain P-L., Bousquet-Mélou A., Damborg P., Ferran A.A., Mevius D., Pelligand L., Veldman K.T. and Lees P. 2017. En Route towards European Clinical Breakpoints for Veterinary Antimicrobial Susceptibility Testing: A Position Paper Explaining the VetCAST Approach. Front. Microbiol. 8:2344. doi: 10.3389/fmicb.2017.02344 17. Zaman S.B., Hussain M.A., Nye R., Mehta V., Mamun K.T., Hossain N. 2017. A Review on Antibiotic Resistance: Alarm Bells are Ringing. Cureus., 9(6):e1403.

OREGANO ESSENTIAL OIL ACCELERATES RECOVERY AND PREVENTS SERUM BIOCHEMICAL IMBALANCES IN CALVES WITH NEONATAL DIARRHEA SYNDROME

Panagiotis D. Katsoulos[1*], Anna Dedousi[2], Maria A. Karatzia[3], Serafeim Chaintoutis[1], Chrysostomos Dovas[1], Elias Papadopoulos[1], Labrini V. Athanasiou[4]

[1]Aristotle University of Thessaloniki, School of Veterinary Medicine, Thessaloniki, Greece
[2]Veterinary Research Institute, Hellenic Agricultural Organization – Dimitra, Thermi, Thessaloniki, Greece
[3]Research Institute of Animal Science, Hellenic Agricultural Organization – Dimitra, Paralimni Giannitsa, Greece
[4]University of Thessaly, Faculty of Veterinary Medicine, Karditsa, Greece
[*]e-mail: katsoulo@vet.auth.gr

Abstract

This study investigated whether the daily administration of oregano essential oil could diminish the severity of calves' neonatal diarrhea syndrome and ameliorate the observed serum biochemical imbalances. Ninety-one newborn calves were randomly assigned into two groups; "Eco" group (n=46) calves were drenched with oregano essential oil (ECODIAR® liquid 5%; 12.5 mg Kg^{-1} body weight once per day) until the age of 10 days. "Conts" group (n=45) calves were left untreated and served as controls. Fecal samples were collected on days 3, 6 and 10 for microbiological and parasitological evaluation. All animals were monitored daily for the incidence of diarrhea until the age of 15 days and their fecal score was recorded. Diarrheic calves were clinically evaluated daily and treated with oral rehydrating solutions. Calves with severe dehydration, sings of acidosis or septicemia received advanced treatment. Blood samples, collected at the age of 48h and 24h after recovery from diarrheic calves that did not receive advanced treatment, were used for biochemical analysis. Average fecal score throughout the experiment, incidence of diarrhea, duration and severity of diarrhea episodes and number of animals that needed advanced treatment were significantly lower in Eco group compared to Conts. Serum concentrations of albumin, BUN, creatinine, K, and P were significantly lower and those of Ca was significantly higher in Eco group than in Conts group. Daily administration of oregano essential oil until the age of 10 days diminishes the severity of diarrhea and prevents the impairment of renal function and electrolyte imbalances

of diarrheic calves.

Key words: oregano essential oil, calves, neonatal diarrhea, treatment, recovery, serum biochemicals

Introduction

Neonatal calf diarrhea syndrome is the most common cause of illness and mortality in calves aged less than 15 days (De la Fuente et al., 1999; Berragry, 1997). The primary infectious agents associated with the disease are enterotoxic *Escherichia coli* (ETEC) K99/F5, *Rotavirus A* (RVA), *Bovine coronavirus* (BCoV), and *Cryptosporidium* spp. (Bartels et al., 2010; Silverlås et al., 2010; Gulliksen et al., 2009) that either alone or in combination directly affect the intestinal epithelial cells, resulting in diarrhea (Cho and Yoon, 2014). Regardless of the primary causative agents, secondary small intestinal overgrowth of *E. coli* bacteria is often observed in diarrheic calves; this is important since increases the severity of diarrhea, retards the recovery, and increases the risk of bacteremia and death (Constable, 2004). Acute diarrhea, apart from decreasing the absorption of essential nutrients from milk, leading to weight loss (Tóthová et al., 2016), is followed by excessive loss of water and electrolytes resulting in dehydration, pre-renal azotemia (Singh et al., 2014) and electrolyte imbalances (Foster and Smith, 2009) that may still be present even 10 days after recovery (Berchtold, 2009), delaying the complete recovery or even causing death.

It is expected that treating diarrheic calves with agents, other than antibiotics, that inhibit coliform bacterial overgrowth in calves with diarrhea might have beneficial effects on the disease outcome by decreasing the duration and the severity of the disease and by preventing the biochemical imbalances and the development of bacteremia. Oregano essential oil could be used as such an antibiotic alternative for this purpose due to its strong antibacterial activity against Gram-negative bacteria and especially *E. coli* (Nazzaro et al., 2013; Si et al., 2008; Elgayyar et al., 2001). Apart from its antibacterial properties, oregano essential oil was also found to have antiviral (Pilauet al., 2011) and anticryptosporidial (Gaur et al., 2016) effects *in vitro*.

The objective of the present study was to evaluate under field conditions whether daily administration of oregano essential oil is effective in diminishing the severity of neonatal diarrhea syndrome and ameliorate the observed serum biochemical imbalances in calves aged less than 15 days.

Materials and methods

Ninety-one newborn Holstein calves (48 females and 43 males) with adequate passive transfer (total proteins > 52 g L^{-1} as determined 48h after birth) from three dairy farms were finally used in the study. They were all born to dams that were vaccinated against rotavirus, coronavirus and *E. coli* F5 (K99)/F41 antigens (Lactovac C; Zoetis, Hellas) one month prior to the expected day of calving. The calves were randomly alternately assigned into one of two groups according to their birth date in each farm. The experiment was run in all 3 farms simultaneously. The animals of Eco group (n=46) were orally drenched with Greek oregano (*Origanum vulgare* ssp. *hirtum*) essential oil (ECODIAR® liquid 5%; Ecopharm Hellas S.A., Kilkis, Greece) at the dose of 12.5 mg Kg^{-1} body weight once per day for the first 10 days of their life whereas those of Conts group (n=45) were left untreated and served as controls.

The experiment started at the day of calving (day 1) and lasted until day 15. Each calf was separated from its dam after calving, weighed, its navel was disinfected using an antibiotic spray (TERRAMYCIN™ AEROSOL SPRAY; Zoetis, Hellas) and was offered its first colostrum meal. The calves of Eco group were orally drenched immediately afterwards with the respective amount of oregano essential oil that was diluted with normal saline up to the volume of 60 ml with the aid of a feeding syringe of equal volume. The following days and until day 10, oregano essential oil was administered in the calves of this group at the same way after the morning feeding.

Feces were blindly scored every day throughout the experiment after the morning feeding by the same person using a three-point scale (1=normal, 3=watery). Calves with scores ≥2 were considered diarrheic. Based on these records, number of days with diarrhea (fecal score ≥2) was determined and diarrhea index (DI) was calculated: DI = number of days with diarrhea x average fecal score on these days.

Fecal samples were collected directly from the rectum on days 3, 6 and 10 of the experiment for microbiological and parasitological evaluation as described previously (Katsoulos et al., 2017). On the same days, the calves of Eco group were weighed, and the daily dosage of oregano essential oil was modified accordingly.

All calves were blindly examined routinely by the same person at the fecal sampling days (days 3, 6 and 10) and at the end of the experiment (day 15). In cases of diarrhea the animals were clinically evaluated daily until recovery. Diarrheic calves in all farms continued milk feeding and were receiving an extra meal per day with oral electrolytes (Diaproof K®; Virbac, Hellas). In cases of severe dehydration or inappetence the calves were receiving Lactated Ringers solution intravenously and, if blood serum glucose concentration was lower than 3 mmol L^{-1} (as determined on farm with a handheld glucose meter; FreeStyle Precision, Abbott, UK), a dextrose 10%

solution was also administered intravenously. Calves with inappetence, hypoglycemia, depression and fever or hypothermia were also treated with antibiotics, based on antimicrobial susceptibility tests the last six months (Farm 2: enrofloxacin 5mg Kg^{-1} SC, Baytril®, Bayer Animal Health GmbH, Germany; Farms 1 and 3: ceftiofour 2.2 mg Kg^{-1} SC, Excenel® RTU, Zoetis, Hellas) and non-steroid anti-inflammatory drugs (carprofen 1.4 mg Kg^{-1} SC, Rimadyl® Cattle, Zoetis, Hellas). In calves of Eco group that were still diarrheic after day 10, administration of oregano essential oil continued until recovery.

Blood serum samples were collected from all calves by jugular venipuncture, 48h after birth (D3) and 24h after recovery (D1PD) from those that did not receive advanced treatment. Serum concentrations of albumin (Alb), urea nitrogen (BUN), creatinine, glucose, total calcium (Ca), inorganic phosphorus (P) and the serum activities of aspartate aminotransferase (AST) and creatine kinase (CK), as well as potassium and sodium concentration were determined in an automated chemistry analyzer (ADVIA 1800 Clinical Chemistry System, Siemens Healthineers).

Data were analyzed using the statistical software JASP 16.1. Normality of data distribution was assessed with Shapiro-Wilk test and homogeneity of variances was evaluated with Levene test. Chi-square test was used to determine whether the incidence of diarrhea and the percentage of animals that needed advanced treatment were significantly different among groups. The effects of the fixed factors group, Farm, detected pathogens, as well as of their interactions, on the fecal score, the number of days with diarrhea, the fecal score at the days with diarrhea and the diarrhea index were tested with Univariate Analysis of Variances. Repeated measures ANOVA was run to evaluate the effect of sampling day (day) and of oregano essential oil administration (group) on the biochemical parameters evaluated. In both models Post-hoc comparisons were done with Bonferroni test. Kaplan-Meier survival analysis (MedCalc Software 9.2.1; MedCalc, Ostend, Belgium) was performed for plotting the time needed for the diarrheic calves to recover after the onset of clinical signs at each group and for the comparison of the obtained curves. A value of $p \leq 0.05$ was considered significant in all comparisons.

Results

The infectious agents related with neonatal diarrhea syndrome that were detected at repeated fecal samplings are presented in Table 1. The percentage of animals found positive for RVA and *Cryptosporidium* spp. was not significantly different between groups ($p>0.05$) or farms ($p>0.05$). BCoV was detected only in Farm 1 and the percentage of positive animals was similar among groups ($p>0.05$).

Table 1. Incidence of diarrhea, and percentages of positive calves to *Cryptosporidium spp.*, *Rotavirus A* (RVA), *Bovine coronavirus* (BCoV) and ETEC K99/F5 in the three farms of the experiment (Farm 1, 2 and 3) and in calves drenched with oregano essential oil (Eco) or left untreated as controls (Conts)

			Eco	Conts
Diarrhea incidence (%)	***Overall***	***80.2***	***69.6***[a]	***91.1***[b]
	Farm 1	71.0	50.0[a]	93.3[b]
	Farm 2	80.0	80.0[a]	80.0[a]
	Farm 3	90.0	80.0[a]	100.0[a]
Cryptosporidium spp. positive (%)	***Overall***	***68.1***	***60.9***[a]	***75.6***[a]
	Farm 1	74.2	68.8[a]	80.0[a]
	Farm 2	70.0	60.0[a]	80.0[a]
	Farm 3	60.0	53.3[a]	67.7[a]
RVA positive (%)	***Overall***	***91.2***	***89.1***[a]	***93.3***[a]
	Farm 1	87.1	81.3[a]	93.3[a]
	Farm 2	100.0	100.0[a]	100.0[a]
	Farm 3	86.7	86.7[a]	86.7[a]
BCoV positive (%)	***Overall***	***7.7***	***8.7***[a]	***6.7***[a]
	Farm 1	22.6	25.0[a]	20.0[a]
	Farm 2	0	0[a]	0[a]
	Farm 3	0	0[a]	0[a]
ETEC positive (%)	***Overall***	***0.0***	***0.0***[a]	***0.0***[a]
	Farm 1	0	0[a]	0[a]
	Farm 2	0	0[a]	0[a]
	Farm 3	0	0[a]	0[a]

[a,b]Different superscripts at the same row denote significant difference (p<0.05)

Diarrhea was recorded in 73 out of the 91 calves (80.2%) (Table 1). The incidence of diarrhea was significantly lower (p<0.05) in calves receiving oregano oil (Eco group) compared to the controls (p<0.05; Table 1). Within farms, the incidence of diarrhea in Farm 1 was also significantly lower in Eco group than the Conts one (p<0.05; Table 1), whereas in Farms 2 and 3 no significant difference was recorded among groups (p<0.05; Table 1). Thirteen out of 73 (17.8%) diarrheic calves had two episodes of diarrhea during the experimental period, without, however, the percentages being significantly different between groups (25% and 12% for Eco and Conts groups, respectively; p>0.05)

The average age of diarrhea onset was not significantly different between groups (mean±SE: 5.89±0.43 and 6.07±0.45 days for groups Eco and Conts, respectively; p>0.05). In Eco group all diarrhea cases started before day 10 and no new case was recorded between days 10 and 15 of the study. As it is shown in Table 2, average fecal score recorded in calves throughout the experiment, average number of days that calves had diarrhea and average diarrhea index were significantly affected only by the daily administration of oregano oil and were significantly lower in Eco group compared to the controls (p<0.05).

Table 2. Average (mean±SE) fecal score at the 15 days of the study, number of days with diarrhea, fecal score at the days with diarrhea and diarrhea index in calves receiving oregano oil (Eco) and those left untreated (Conts)

		Fecal score	**Days with Diarrhea**	**Diarrhea fecal score**	**Diarrhea Index**
Eco		1.20±0.05	3.86±0.41	2.42±0.06	9.52±1.09
Conts		1.52±0.06	5.18±0.42	2.44±0.06	12.97±1.21
p	*Group*	***	*	NS	*
	Farm	NS	NS	NS	NS
	Pathogen	NS	NS	NS	NS

*p<0.05; ***p<0.001; NS: no significant (p>0.05)

However, fecal score on days with diarrhea was not significantly affected by the group of animals. The percentage of calves that needed advanced treatment was significantly lower (p<0.05) in Eco group than the controls (Table 3). Significantly lower was also the percentage of animals that needed IV fluids and antibiotic treatment in Eco group than the Conts (p<0.05; Table 3).

Table 3. Number and percentage (%) of the 73 diarrheic calves that needed advanced treatment (IV fluids or IV fluids and antibiotics) in the group of animals that received oregano essential oil (Eco) and those left untreated (Conts)

		n	Eco	Conts
Calves needed treatment		***36***	***9 (28.1%)[a]***	***27 (65.9%)[b]***
Treatment	IV fluids	19	6 (31.6%)[a]	13 (53.8%)[a]
	IV fluids & antibiotics	17	3 (17.6%)[a]	14 (82.4%)[b]

[a,b]Different superscripts at the same row denote significant difference ($p<0.05$)

The survival curves depicting the time until recovery at diarrhea episodes in both groups are presented in Figure 1.

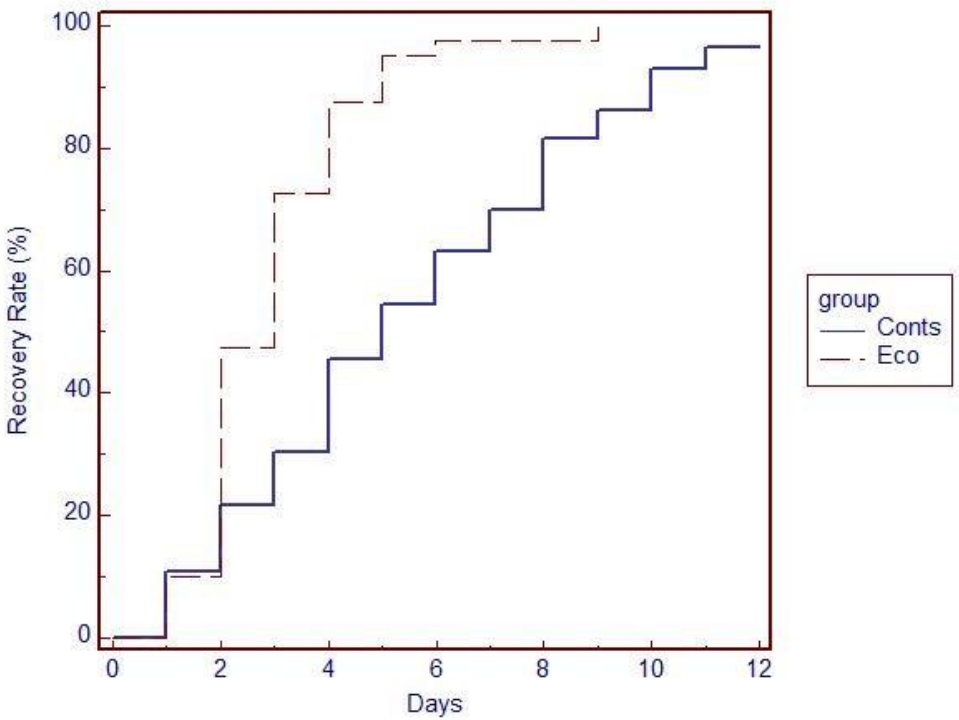


Figure 1. Kaplan-Meier survival curve depicting the time needed for recovery after the onset of diarrhea at the 32 diarrheic calves in the group of animals receiving oregano essential oil (Eco) and at the 41 diarrheic calves in the control group (Conts)

The analysis revealed that the median time until recovery was significantly lower ($p<0.05$) in Eco group (3 days) compared to the controls (5 days). No death was recorded in any experimental group.

The biochemical alterations of calves that did not receive advanced treatment are presented in Table 4 and Figure 2. A significant increase of serum creatinine concentration between D3 and D1PD was observed within Conts group ($p<0.05$; Figure 2A) whereas this increase was insignificant in Eco group ($p>0.05$; Figure 2A). The average values recorded on D1PD were significantly lower in Eco group compared to the controls ($p<0.05$; Figure 2A). On D1PD, 4 out of 14 (28.57%) animals of Conts group and 1 out of 23 (4.35%) calves of Eco group had serum creatinine values higher than the upper reference range (133 umol L^{-1}) but the difference was not significant ($p>0.05$). However, the percentage of animals with creatinine concentration higher than 100 umol L^{-1} was significantly higher ($p<0.05$) in Conts group (57.14%) than Eco group (17.39%). BUN concentration was significantly higher on D1PD in comparison with D3 within Conts group ($p<0.05$) but not within Eco group ($p>0.05$). In addition, on D1PD BUN was significantly higher in Conts group than Eco ($p<0.05$; Figure 2b). On this day, BUN values higher than the upper reference limit (10.9 mmol L^{-1}) was observed on the same calves (4 in Conts group and 1 in Eco group) that had high serum creatinine concentration in both groups.

Table 4. Mean±SE of biochemical parameters in calves drenched with oregano essential oil (Eco) and those left untreated (Conts) 48h after birth (D3) and 24h after recovery from diarrhea (D1PD).

	ECO	Conts	D3	D1PD	Reference range
Alb ($g\ L^{-1}$)	32.78 ± 0.79^{a}	35.30 ± 0.79^{b}	32.56 ± 0.63^{A}	35.50 ± 0.63^{b}	25 – 37
Glucose ($mmol\ L^{-1}$)	5.19 ± 0.21^{a}	5.16 ± 0.21^{a}	5.27 ± 0.24^{A}	4.98 ± 0.24^{A}	3.9 – 8.5
BUN ($mmol\ L^{-1}$)	4.47 ± 0.52^{a}	5.27 ± 0.52^{a}	3.71 ± 0.50^{A}	6.03 ± 0.55^{B}	2.7 – 10.9
Creatinine ($umol\ L^{-1}$)	76.58 ± 3.59^{a}	96.42 ± 3.59^{b}	76.81 ± 3.19^{A}	96.19 ± 3.19^{B}	56 – 133
Ca ($mmol\ L^{-1}$)	2.44 ± 0.04^{a}	2.32 ± 0.04^{b}	2.47 ± 0.04^{A}	2.29 ± 0.04^{B}	2.0 – 3.0
P ($mmol\ L^{-1}$)	2.05 ± 0.06^{a}	2.21 ± 0.06^{a}	2.03 ± 0.05^{A}	2.22 ± 0.05^{B}	1.3 – 2.6
K ($mEq\ L^{-1}$)	5.30 ± 0.14^{a}	5.82 ± 0.14^{b}	5.27 ± 0.13^{A}	5.85 ± 0.13^{B}	3.5 – 5.5
Na ($mEq\ L^{-1}$)	134.88 ± 0.67^{a}	134.72 ± 0.67^{a}	135.30 ± 0.58^{A}	134.29 ± 0.58^{A}	132 – 152
AST ($U\ L^{-1}$)	45.31 ± 2.76^{a}	52.34 ± 2.76^{a}	52.72 ± 2.61^{A}	43.93 ± 2.61^{B}	19 – 55
CK ($U\ L^{-1}$)	94.48 ± 27.52^{a}	136.84 ± 27.52^{a}	144.90 ± 27.11^{A}	86.42 ± 27.11^{A}	0 – 400

^{A,B,a,b}Different superscripts between groups (a,b) or between days (A,B) denote significant difference at $P\leq0.05$; Alb: albumin; BUN: blood urea nitrogen; Ca: total calcium; P: inorganic phosphorus; K: potassium; Na: sodium; AST: aspartate aminotransferase; CK: creatine kinase.

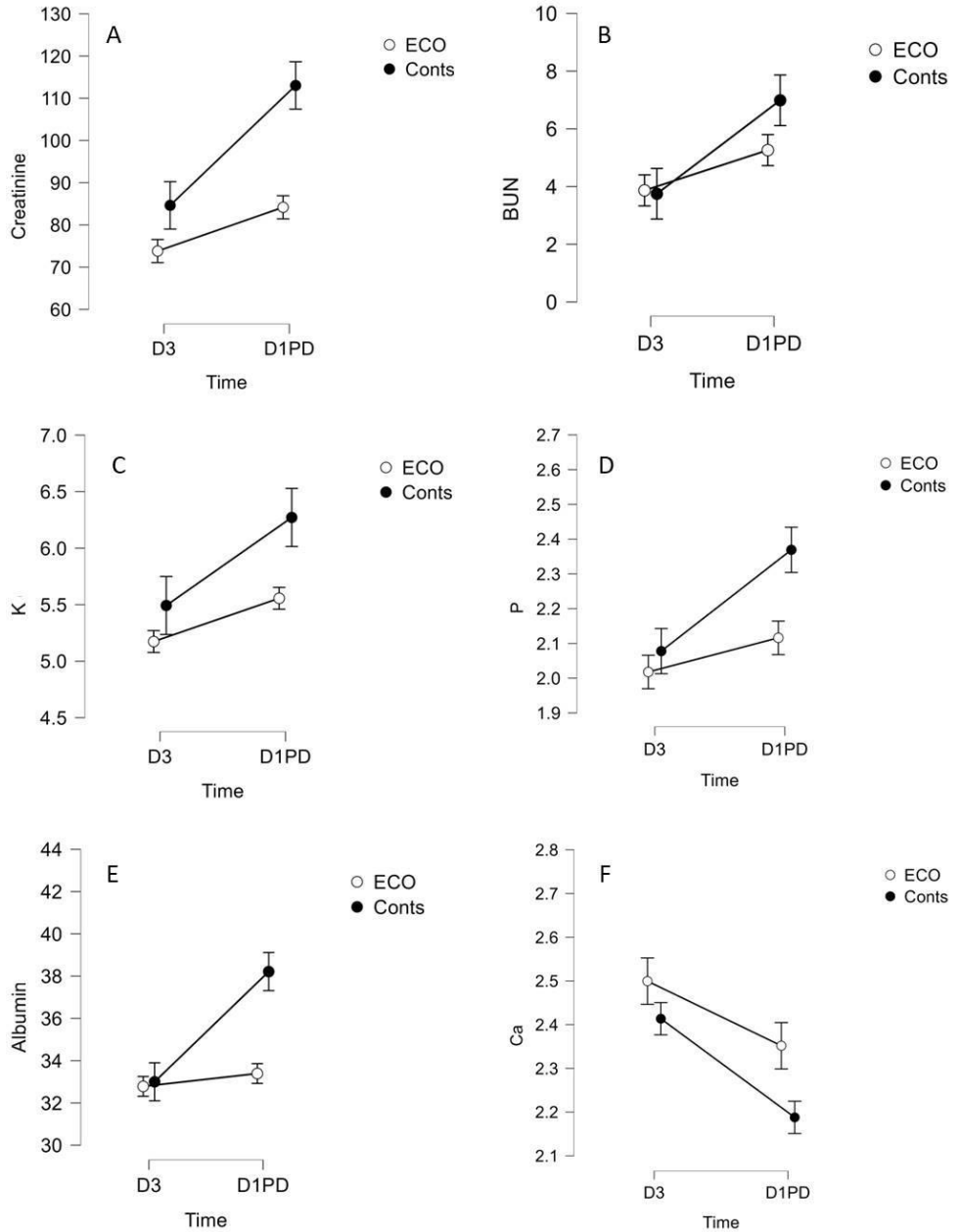


Figure 2. Mean±SE of serum concentrations of A: creatinine (umol L^{-1}), B: blood urea nitrogen (mmol L^{-1}), C: potassium (K; mEq L^{-1}), D: inorganic phosphorus (P; mmol L^{-1}), E: albumin (g L^{-1}) and F: total calcium (Ca; mmol L^{-1}) in the group of animals that received oregano essential oil (Eco) and those left untreated (Conts) 48h after birth (D3) and 24h after recovery from diarrhea (D1PD).

Serum K and P concentrations were significantly higher on D1PD compared to D3 ($p>0.05$; Table 4); this increase was significant only within Conts group ($p<0.05$; Figures 2C and 2D). On D1PD, the average values recorded in Eco group were significantly lower ($p<0.05$) compared to the controls for both parameters (Figures 2C and 2D).

Serum Alb was significantly lower in ECO group compared to the controls and in D3 than D1PD whereas serum Ca was significantly higher than Conts in both comparisons (Table 4). As it is shown in Figure 2, serum Alb was increased significantly ($p<0.05$; Figure 2E) and serum Ca was decreased significantly ($p<0.05$; Figure 2F) within Conts group, and their concentrations were significantly different among groups on D1PD ($p<0.05$). Concerning the activities of the enzymes evaluated, AST values recorded were significantly reduced between D3 and D1PD ($p<0.05$; Table 4). However, the alterations observed within both groups were not significant ($p<0.05$). Serum activity of CK remained practically stable between D3 and D1PD ($p<0.05$; Table 4).

Discussion

The incidence of diarrhea at the present experiment was significantly lower in calves drenched with oregano essential oil compared to the controls. However, this trend was not observed in all farms but only in one. So, it cannot be supported with certainty that oregano oil administration has preventive effect against neonatal diarrhea syndrome; it could be possibly assumed that this effect might be possible in farms with intensive hygiene management practices where calves have lower challenge of infectious agents.

The significantly lower number of days with diarrhea, the shorter duration of the diarrhea episodes, the lower diarrhea index and the lower number of calves needed advanced treatment in Eco group compared to the controls, indicate that daily administration of oregano essential oil can effectively diminish the severity of neonatal diarrhea syndrome in calves. Research evidence from other animal species indicate that oregano essential oil has a protective effect on intestinal mucosa by modifying the intestinal microflora, inhibiting the expression of inflammatory cytokines and enhancing its antioxidative capacity (Zou et al., 2016; Wei et al., 2015). In our case, it is believed that oregano essential oil inhibited the coliform bacterial overgrowth in the small intestine of diarrheic calves due to its strong antibacterial activity against *E. coli* (Nazzaro et al., 2013; Si et al., 2008; Elgayyar et al., 2001). In support to this point of view, it was observed that oregano essential oil drenching had comparable results with neomycin administration per os on the treatment outcome of

diarrhea due to *E. coli* in calves (Bampidis et al., 2005). Furthermore, the significantly lower number of calves with systemic illness that needed antimicrobial and supportive therapy in Eco group, which is suggestive of lower cases of bacteremia (Constable, 2004), provides further evidence that oregano essential oil might inhibit intestinal coliform overgrowth.

The results of serum biochemical analysis in calves that did not receive advanced treatment indicate that oregano essential oil protects diarrheic calves from pre-renal azotemia and severe electrolyte imbalances in the context of treatment management followed at this study. Azotemia is attributed to hypovolemia and the consequent reduced renal blood flow and glomerular filtration rate due to dehydration (Singh et al., 2014). The significantly lower incidence of azotemia along with the alterations for BUN and creatinine, prove that calves receiving oregano oil were able to maintain a more efficient glomerular filtration rate compared to the controls. It further indicates that control calves were more severely dehydrated during the disease than those of Eco group; this is confirmed by the significant increase of serum Alb concentration only in Conts group and the significantly higher serum Alb concentration on D1PD recorded in this group. The significant increase of serum K and P during diarrhea and the significantly higher values of these macroelements recorded in the control compared to the Eco group are attributed to the hypovolemia and the impaired renal function of the calves of this group. It is well documented that the increase in proximal tubular reabsorption of water and sodium along with the decreased glomerular filtration rate due to hypovolemia result in reduced distal tubular K secretion and, consequently, in increased K concentration in calves with prerenal azotemia (Trefz et al., 2013). Increased P concentration is usually observed in calves with hyperkalemia; it is mainly due to dehydration and prerenal azotemia but it is referred that acute acidemia might also be responsible for part of the increased P concentration (Trefz et al., 2013).

The decrease in serum Ca concentration during diarrhea is attributed to calcium loss with feces (Lewis and Phillips, 1972). However, the decline was significant only in the control group; this finding probably indicates that diarrhea and, consequently, the fecal loss of Ca was more severe in this group. Serum glucose and Na concentrations remained unaffected by both diarrhea and the administration of oregano essential oil. This is in contrast with other findings supporting that, diarrheic calves, have lower concentrations of glucose and Na (Singh et al., 2014; Özkan et al., 2011; Seifi et al., 2006) than the non-diarrheic ones. However, all calves were offered an oral rehydrating solution that contained an energy source and Na twice a day which protected the diarrheic calves from hypoglycemia and hyponatremia.

The serum activity of AST was significantly lower after diarrhea compared to D3, and the activity of CK remained practically stable. This corresponds to the normal variation of these enzymes for calves of this age (Egli and Blum, 1998) and indicates that diarrhea is not associated either with liver or muscle damage. It further provides evidence that liver function is not impaired by the administration of oregano essential oil in diarrheic neonatal calves.

Conclusions

In the context of this study, it can be concluded that daily administration of Greek oregano essential oil in calves for the first 10 days of their life effectively diminishes the severity of naturally acquired diarrhea under field conditions prevents the impairment of the glomerular filtration rate and the electrolyte imbalances of diarrheic calves.

Acknowledgements

This research has been funded by the Research Committee of Aristotle University of Thessaloniki (grant number 93297)

Authors' contributions

PDK: Conception, study design, data collection, analysis and interpretation, manuscript drafting; AD, MAK: study design, data collection and interpretation, manuscript critically revising; SC, CD, EP, LVA: study design, sample analysis, data interpretation, manuscript critically revising. All authors approved the final version of the manuscript.

Conflict of interest

The authors have no conflict of interest to declare

References

1. Bampidis V. A., Christodoulou V., Florou-Paneri P., Christaki E. 2006. Effect of dried oregano leaves versus neomycin in treating newborn calves with colibacillosis. Journal of Veterinary Medicine series A - Physiology Pathology Clinical Medicine, 53:154-156. 2. Bartels C. J. M., Holzhauer M., Jorritsma R., Swart W. A. J. M., Lam T. J. G. M. 2010. Prevalence, prediction and risk factors of enteropathogens in normal and non-normal faeces of young Dutch dairy calves. Preventive Veterinary Medicine, 93:162–169. 3. Berchtold J. 2009. Treatment of calf diarrhea: intravenous fluid therapy. Veterinary Clinics of North America: Food Animal Practice, 25:73-99, vi. 4. Berragry T. 1997. Calf diarrhoea. Irish Veterinary Journal, 50:49-58. 5. Cho Y. I., Han J. I., Wang C., Cooper V., Schwartz K., Engelken T., Yoon K. Y. 2013. Case-control study of microbiological etiology associated with calf diarrhea. Veterinary Microbiology, 166:375—385. 6. Constable P. D. 2004. Antimicrobial use in the treatment of calf diarrhea. Journal of Veterinary Internal Medicine, 18:8-17. 7. de la Fuente R., Luzón M., Ruiz-Santa-Quiteria J. A., García A., Cid D., Orden J. A., García S., Sanz R., Gómez-Bautista M. 1999. Cryptosporidium and concurrent infections with other major enterophatogens in 1 to 30-day-old diarrheic dairy calves in central Spain. Veterinary Parasitology, 80:179-185. 8. Egli C. P., Blum J. W. 1998. Clinical, Haematological, Metabolic and Endocrine Traits During the First Three Months of Life of Suckling Simmentaler Calves Held in a Cow-Calf Operation. Journal of Veterinary Medicine series A - Physiology Pathology Clinical Medicine, 45:99-118. 9. Elgayyar M., Draughon F. A., Golden D. A., Mount J. R. 2001. Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms.

Journal of Food Protection, 64:1019–1024. 10. Foster D. M., Smith G. W. 2009. Pathophysiology of diarrhea in calves. Veterinary Clinics of North America: Food Animal Practice, 25:13-36, xi. 11. Gaur S., Kuhlenschmidt T. B., Kuhlenschmidt M. S.,AndradeJ E. 2016. Oregano Essential Oil and Carvacrol reduce Cryptosporidium parvum infectivity of HCT-8 Cells. The FASEB Journal, 30(1 Suppl):668.12. 12. Gulliksen S. M., Jor E., Lie K. I., Hamnes I. S., Løken T., Åkerstedt J., Østerås O. 2009. Enteropathogens and risk factors for diarrhea in Norwegian dairy calves. Journal of Dairy Science, 92:5057–5066. 13. Katsoulos P. D., Karatzia M. A., Dovas C. I., Filioussis G., Papadopoulos E., Kiossis E., Arsenopoulos K., Papadopoulos T., Boscos C., Karatzias H. 2017. Evaluation of the in-field efficacy of oregano essential oil administration on the control of neonatal diarrhea syndrome in calves. Research in Veterinary Science, 115:478-483. 14. Lewis L. D., Phillips R. W. 1972. Water and electrolyte losses in neonatal calves with acute diarrhea. A complete balance study. The Cornell Veterinarian, 62:596-607. 15. Nazzaro F., Fratianni F., De Martino L., Coppola R., De Feo V. 2013. Effect of Essential Oils on Pathogenic Bacteria. *Pharmaceuticals, 6:*1451-1474. 16. Pilau M. R., Alves S. H., Weiblen R., Arenhart S., Cueto A. P., Lovato L. T. 2011. Antiviral activity of the Lippiagraveolens (Mexican oregano) essential oil and its main compound carvacrol against human and animal viruses. Brazilian Journal of Microbiology, 42:1616–1624. 17. Si H., Hu J., Liu Z., Zeng Z. L. 2008. Antibacterial effect of oregano essential oil alone and in combination with antibiotics against extended-spectrum β-lactamase-producing *Escherichia coli*. FEMS Immunology and Medical Microbiology, 53:190–194. 18. Silverlås C., de Verdier K., Emanuelson U., Mattsson J. G., Björkman C. 2010. *Cryptosporidium* infection in herds with and without calf diarrhoeal problems. Parasitology Research, 107:435–1444. 19. Singh M., Gupta V., Mondal D., Bansal S., Sharma D., Shakya M. 2014. A study on alteration in Haemato-biochemical parameters in Colibacillosis affected calves. International Journal of Advanced Research, 2:746-750. 20. Tóthová C., Nagy O., Kováč G., Nagyová V. 2016. Changes in the concentrations of serum proteins in calves during the first month of life. Journal of Applied Animal Research, 44:338-346. 21. Trefz F. M., Lorch A., Feist M., Sauter-Louis C., Lorenz I. 2013. The prevalence and clinical relevance of hyperkalaemia in calves with neonatal diarrhoea. The Veterinary Journal, 195:350-356.

ANTIMICROBIAL RESISTANCE IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM PIGS FROM MLADENOVAC, SERBIA

Vuk Vračar[1*], Jana Mitrović[1], Gordana Kozoderović[2], Milan Đorđević[3], Ivan Galić[1], Vesna Lalošević[1]

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[2]*University of Novi Sad, Faculty of Education in Sombor, Sombor, Serbia*

[3]*Veterinary station "Mladenovac", Mladenovac, Serbia*

[*]*e-mail: vuk.vracar@polj.uns.ac.rs*

Abstract

Antimicrobial resistance (AMR) is a major challenge for both human and veterinary medicine. It is estimated that more than 700.000 death cases annually are the result of infection by resistant bacterial strains, and it is expected that this numbers will increase over the years. Due to its ubiquity and high capacity to accumulate AMR genes *Escherichia coli* represents a major reservoir for these genes. Data on this topic regarding the pigs on the territory of the Republic of Serbia are scarce, the aim of this study was to determine the presence of antibiotic resistance in pig fecal isolates using disc-diffusion method, and to examine the presence of *bla*NDM gene using conventional PCR.

For the purpose of this study, 62 pooled stool samples from pigs were collected during 2018 at individual small farms in the area of Mladenovac municipality. For the antimicrobial susceptibility testing of the obtained 59 *Escherichia coli* isolates the disc diffusion method was used according to EUCAST guidelines. The highest resistance was observed to ampicillin (28.8%), and the lowest resistance to cefuroxime and ciprofloxacin (1.7%), while none of the isolates were resistant to ceftriaxone. Extended-spectrum β-lactamases (ESBL) and multidrug resistance (MDR) were found in one sample each (1.7%). All of the strains were negative for the presence of carbapenemase encoded gene *bla*NDM, respectively.

Both the low AMR rates and the absence of *bla*NDM gene in samples tested in this research could be an effect of less frequent use of antibiotic on small-scale farms, that is conditioned by less disease pressure and lower stock pig density in this type of pig farms.

Key words: antimicrobial resistance, disc-diffusion method, pigs, *bla*NDM gene, Serbia

Sažetak

Antimikrobna rezistencija (AMR) predstavlja veliki izazov i za humanu i za veterinarsku medicinu. Procenjuje se da je više od 700.000 smrtnih slučajeva godišnje rezultat infekcije rezistentnim bakterijskim sojevima, a očekuje se da će se taj broj povećavati tokom godina. Zbog svoje sveprisutnosti i velikog kapaciteta za akumulaciju AMR gena, Escherichia coli predstavlja glavni rezervoar ovih gena. Podaci u vezi sa svinjama na teritoriji Republike Srbije na ovu temu su oskudni, te je cilj ovog istraživanja bio da se disk- difuzionim metodom utvrdi prisustvo rezistencije na antibiotike kod izolata E. coli iz fecesa svinja, kao i da se ispita prisustvo blaNDM gena primenom konvencionalnog PCR-a. Za potrebe ove studije tokom 2018. Godine prikupljena su 62 pulirana uzorka stolice svinja s pojedinačnih malih farmi s područja opštine Mladenovac. Za ispitivanje antimikrobne osetljivosti dobijenih 59 izolata Escherichia coli korišćen je disk-difuzioni metod prema smernicama EUCAST-a. Najveća rezistencija uočena je prema ampicilinu (28,8%), a najmanja prema cefuroksimu i ciprofloksacinu (1,7%), dok nijedan izolat nije bio rezistentan na ceftriakson. Beta-laktamaze proširenog spektra (ESBL) i multipla rezistencija (MDR) pronađene su u po jednom uzorku (1,7%). Svi sojevi su bili negativni na prisustvo karbapenemaze kodirane blaNDM genom. Niske stope AMR i odsustvo blaNDM gena u uzorcima testiranim u ovom istraživanju mogli bi da budu posledica ređe upotrebe antibiotika na malim farmama, što je uslovljeno manjim opterećenjem bolestima i nižom gustinom naseljenosti kod ove vrste farmi svinja.

Ključne reči: antimikrobna rezistencija, disk-difuziona metoda, svinje, blaNDM gen, Srbija

Introduction

Emergence and increase in numbers of bacterial strains resistant or multiresistant to antibiotics are one of the major health challenges of 21st century because it bears negative effects to health which are reflected in failure or prolongation of the therapy and in fatal outcomes of chronic cases. According to the WHO, at least 700,000 people die every year as a result of infection caused by resistant strains and it is expected that it will exceed 10,000,000 by 2050 (Na et al., 2018). With between 50-70% of all known antibiotic resistance incidences β-lactamases (*bla*) are the largest class of antibiotic resistance genes (Xiaocao et al., 2022). Among the drug-resistant bacteria of global importance *Escherichia coli*

especially stands out, not only not as one of the most significant causes of foodborne infections, but as a major reservoir of antimicrobial resistance genes. This is due to the widespread and common existence of *E. coli* and drug-resistant plasmids and the high capacity of *E. coli* to accumulate these genes most often through the horizontal gene transfer (Peng et al., 2022; Li et al., 2021). As the last-resort antibiotics, carbapenems have been used for the treatment of infections caused by extended spectrum β-lactamase-producing *Enterobacteriaceae, E. coli* and *K. pneumoniae*. The higher frequency of such strains has led to higher carbapenem usage which has resulted in the emergence and spread of carbapenemase-producing *Enterobacteriaceae*. The most common carbapenem resistance among the carbapenem-resistant clinical isolates of *Enterobacteriaceae* is a production of carbapenemases: NDM, OXA-48-like, and KPC, respectively (Han et al., 2020; Murugan et al., 2019).

More than half of all antimicrobial use occurs in the animal industry for treatment or as feed additives for infection prophylaxis and growth promotion, thus this overuse of antimicrobial agents in livestock is one likely initiator of the high AMR burden (Pholwat et al., 2020). Antimicrobial resistance in food animals impacts both human and animal health directly by introduction of AMR strains into the food chain, and indirectly by spreading these strains through water and soil contamination. There are clear evidence that the widespread use of oral antimicrobials within pig production was a significant risk factor for development of AMR and even multidrug resistant (MDR) *E. coli* (Sirichokchatchawan et al., 2021). On the other hand, recently conducted studies have shown that stopping the use of antimicrobials in feed is associated with decreased rates of AMR (Pholwat et al., 2020). Despite religious restrictions in many countries pig farming is the animal industry that experiences the strongest growth with an expected increase of production of 8.6% by 2030 and 12.7% by 2050 projected by the Food and Agriculture organization of the United Nations (Monger et al., 2021). Above mentioned data unequivocally indicate a close relationship between AMR and pig farming, therefore, it is to be expected that the number of AMR strains will increase in parallel with the increase in pig production.

Considering the importance of AMR and the fact that the data on this topic regarding the pigs from the territory of the Republic of Serbia are scarce, the aim of this study was to determine the presence of antibiotic resistance in pig fecal *E. coli* isolates using disc-diffusion method, and to examine the presence of *bla*NDM gene using conventional PCR.

Material and methods

For the purpose of this research 62 pooled samples of pig feces were collected during the 2018 on individual small farms in the area of Mladenovac municipality. Using the standard bacteriological methods 59 *E. coli* strains was isolated from pig feces, while identity of the strains was determined by standard biochemical methods. Antimicrobial susceptibility was performed using the Kirby-Bauer method, the disk diffusion method respectively, according to the guidelines of the Institute for Clinical and Laboratory Standards and the European Committee on Antimicrobial Susceptibility Testing (CLSI, 2017; EUCAST, 2017). The antimicrobial susceptibility of all *E. coli* isolates was tested for the following antibiotics: ampicillin (10 µg), amoxicillin + clavulanic acid (20 + 10 µg), cefuroxime (30 µg), ceftriaxone (30 µg), gentamicin (10 µg), trimethoprim-sulfamethoxazole (1.25 + 23.75 µg), ciprofloxacin (5 µg). Isolates resistant to 3 or more antibiotic classes were considered multiresistant.

Bacterial DNA was obtained by boiling of suspension of overnight colonies in 1 ml of distilled DNA free water and boiled for 10 at 100º C, and then were centrifuged for 5 minutes at 6000 g. Obtained supernatant was further used as DNA template. In order to determine the presence of *bla*NDM gene in the *E. coli* strains a conventional PCR was done under the conditions previously described by Grubdmann et. al. (2017).

Results

In this study,the 59 obtained *Escherichia coli* isolates from stool samples of pigs were tested for antimicrobial susceptibility. Antimicrobial resistance was noted in 27 samples (45.8%), whereas 32 isolates (54.2%) showed susceptibility to all used antibiotics.

The highest resistance was observed to ampicillin (28.8%), followed by amoxicillin with clavulanic acid (15.3%), sulfamethoxazole with trimethoprim and gentamicin (11.8%), with the lowest resistance to cefuroxime and ciprofloxacin (1.7%). No isolates were resistant to ceftriaxone. The results of the antibiogram are shown in Figure 1.

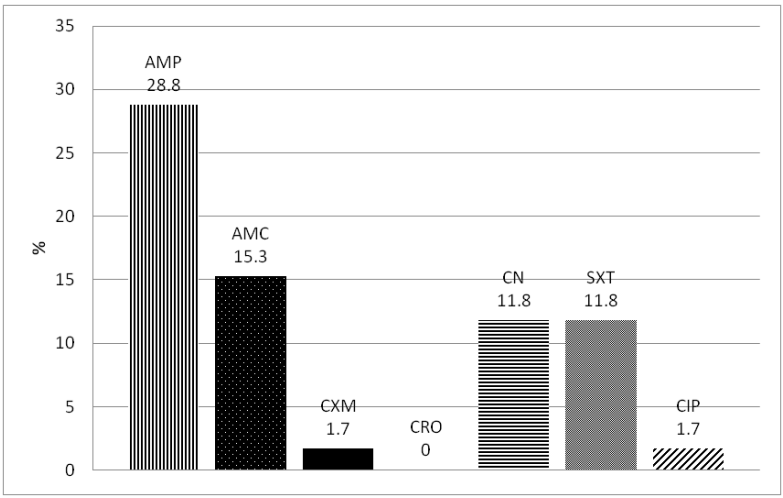


Figure 1. Patterns of antibiotic resistance of *Escherichia coli* isolates. AMP – ampicillin, AMC – amoxicillin with clavulanic acid, CXM – cefuroxime, CRO – ceftriaxone, CN – gentamicin, SXT – sulfamethoxazole with trimethoprim, CIP – ciprofloxacin

Multidrug resistance (MDR), defined as resistance against at least one antibiotic in at least three classes of antibiotics, was found in one (1.7%) isolate of *E. coli*.

Extended-spectrum β-lactamases (ESBL) were noted in one sample (1.7%), i.e. sample 34 resistant to the representatives of penicillin and cephalosporin class of antibiotics (resistant to ampicillin, amoxicillin with clavulanic acid and cefuroxime).

Discussion

Although a National program for the control of antimicrobial resistance was adopted by the Government of the Republic of Serbia for the period 2019-2021, data on this issue are scarce, since a system for the monitoring of antibiotic consumption in animals and control of bacterial resistance to antibiotics in accordance with EU guidelines was not established until 2018 (Krnjaić et al., 2018). Our data shows presence of antibiotic resistance in *E. coli* isolates from pigs, and raises important questions about the potential impact of antibiotic use in food-producing animals and the possible transmission of resistant bacteria to humans through the food chain. Due to the differences in methods applied in studies on AMR, it is difficult to compare prevalence rates of antibiotic resistance obtained in our study with the results of other authors. Speaking in general, the results of our study showed, as it could be expected, the highest AMR rates to the longest used antibiotics – penicillins what is in

correlation with results of other authors, but unlike in a majority of those reports we have found considerably lower rates of resistance to all the tested antibiotics.

In a research conducted in Serbia, in *E. coli* strains originating from pigs a high percentage of resistance only to neomycin or only to gentamicin was found, while considering the related aminocyclotole antibiotic – spectinomycin resistance was determined in all the strains. Resistance to trimethoprim-sulphonamide in healthy pigs was 50%. Although fluoroquinolones are widely used in treating domestic animals, resistance was present only in strains originating from young unhealthy animals (15% to 50%). The resistance in the isolated strains of *E.coli* to enrofloxacin in pigs was 10%. The majority of the isolated *E. coli* strains examined for 17 antibiotics and chemotherapeutics showed resistance to 15, and sensitivity only to cephalosporines of the III generation and colistin (Krnjajić et al., 2005).

The results of Došen at al. from the period 2007-2009 showed highly sensitive to the enrofloxacin (54.84%), neomycin (38.71%), gentamicin (38.71%), lincospectin (29%), flumequine (29%), amoxicillin (26.66%), colistin (17.39%), tetracycline (6.45%), doxycycline (6.45%), streptomycin (3.22%) (Došen et al., 2011).

A significantly higher (64.9%) resistance of *E. coli* isolates to ampicillin, amoxicillin with clavulanic acid (29.7%), sulphamethoxazole with trimetophrim (32.4%) and ciprofloxacin (13.5%) in another study conducted in Serbia was reported, whereas the resistance to gentamicin was lower (2.7%) compared to the results obtained in our study (11.8%). Furthermore, Knezevic and Petrovic found MDR in all of the *E. coli* isolates (100%) from pigs, in contrary to the findings in our study, where MDR was found in only one (1.7%) isolate of *E. coli* (Knezevic and Petrovic, 2008). Ašanin et al. registered resistance to tetracycline and ampicillin in 89.58% isolated strains of *E. coli* in pigs and to ciprofloxacin in 37.5% strains. The lowest percentage of resistance to ceftriaxone was established in 4.17%. The isolated strains of *E. coli* were also resistant to gentamicin, was established in 33.33% strains. All the *E.coli* strains that led to diarrhea in piglets were resistant to at least two antibiotics, and more than 50% strains were found to be resistant to more than 3 antibiotics (Ašanin et al., 2009).

In a research from Croatia, tested strains of *E.coli* in pigs, showed 50% or more resistant to 8 out of 10 tested antimicrobial drugs, and 75% of those were resistant to 4 or more antimicrobials drugs. Results showed the least resistance of tested strains of *E. coli* to amoxicillin with clavulanic acid (9.1%), while resistance to florfenicol was 40.9% and enrofloxacin 50%. Resistance has been established, ranging from 66.7% to gentamicin and cephalexin, 73.3% according to colistin, 80% according to oxytetracycline, 86.2% to flumequine up to 91.3% to sulfamethoxazole

with trimethoprim and 91.4% versus lincomycin with spectinomycin. The results indicate a higher percentage resistant strains compared to previous research in conventional farming pig, and high resistance recorded is also to aminoglycosides (gentamicin) and fluoroquinolones (enrofloxacin), which so far it was not the case (Sukalić et al., 2019).

In contrast to the results of our study, Enne et al. found that 92.1% of the *E. coli* isolates from pigs were resistant to at least one tested antimicrobial, while 62.8% were multi-resistant. Furthermore, 66.9% of the isolates were resistant to trimethoprim-sulphonamide, whereas 25.4% were resistant to ampicillin, and only 1% resistant to gentamicin (Enne et al., 2008).

On the other hand, the prevalence of multi-drug resistant *E. coli* strains was higher (28.8%) in a more recent study from the UK, with similar reports from other countries at the same time, but the resistance to ampicillin, amoxicillin-clavulanic acid and trimethoprim-sulfamethoxazole was much lower than the resistance patterns found in our study, with prevalences 9%, 1%, 5% respectively (Yang et al., 2020).

Mitchaothai and Srikijkasemwat reported that percentages of antimicrobial resistance of overall *E. coli* isolates ranged from lower resistant levels at 23.64% (conventional farming system) and 6.67% (deep litter system) for gentamicin to a very high resistant level as 98.18% for amoxicillin, even 100% for AMC in the conventional farming system (Mitchaothai and Srikijkasemwat, 2022).

Since the first report of a strain of *bla*NDM-1- positive *Klebsiella pneumoniae* from India in 2008, the *bla*NDM-positive *Enterobacteriaceae* have been reported in non-clinical samples India, Pakistan, Bangladesh, Italy, Poland, Denmark, Latin America, and African countries (Chen et al., 2022; Han et al., 2020), yet in our research *bla*NDM gene was not found. Both the lower AMR rates and the absence of *bla*NDM gene samples tested in this research could be explained by the type of pig production system. Small-scale farms, as were included in our research, get less disease pressure as free from constraints of contract farming and lower stock pig density, thus antibiotics are rarely used for disease prevention leading to lower AMR (Mitchaothai and Srikijkasemwat, 2022).

Conclusion

According the results of this study and trends in the world showing increasing bacterial resistance to antimicrobial drugs, fears for the future of treating bacterial infections in pig farms are justified, especially among vulnerable piglet populations before and after weaning. If we look at the results that are related to the susceptibility of isolated *E. coli* strains to antimicrobials, in almost every research so far a high or

very high resistance is notable. A large number of resistant strains was established on the farms where antibiotics have not been used as growth promoters or in the prophylaxis, suggesting that antibiotics are not the only selective factors for antibiotic resistance. The fact that we didn't determine the presence of *bla*NDM gene in our research doesn't exclude it's presence, especially knowing that the samples we tested have been collected during 2018 and it's about a relatively new discovered gene. Considering all the above mentioned, new studies on a larger scale on this topic are needed in order to establish monitoring programs over AMR resistant *E. coli* strains in animal industry and providing guidance for a rational antibiotic use in the future.

Acknowledgements

This research was supported by Ministry of Education, Science and Technological Development (Grant number: 451-03-68/2022-14/200117)

References

1. Ašanin R., Žutić M., Ašanin J., Milić D., Žutić J., Jakić-Dimić D., Milić N., J. N. 2009. Ispitivanje prisustva novih oblika rezistencije na neke antibiotike kod sojeva E. coli izolovanih od prasadi. Veterinarski glasnik, 63:311-120. 2. Chen W., Liu Z., Lin H., Yang J., Liu T., Zheng J., Long X., Sun Z., Li J., Chen X. 2022. Occurrence of blaNDM-1-Positive *Providencia* spp. in a Pig Farm of China. Antibiotics, 11:713. 3. CLSI, 2017. Performance Standards for Antimicrobial Susceptibility Testing. 27th ed. Clinical and Laboratory Standards Institute, Wayne, PA. Accessed on 01.09.2017. 4. Došen R., Prodanov-Radulović J., Pušić I., Stojanov I., Stojanović D., Ratajac R. 2011. Resistance *Escherichia coli* isolates to antibiotics from the organ samples originating from swine farms. Biotechnology in Animal Husbandry, 27:861-866. 5. Enne VI, Cassar C., Sprigings K., Woodward MJ, Bennett PM. 2008. A high prevalence of antimicrobial resistant *Escherichia coli* isolated from pigs and a low prevalence of antimicrobial resistant *E. coli* from cattle and sheep in Great Britain at slaughter. FEMS microbiology letters, 278:193-199. 6. European Committee on Antimicrobial Susceptibility Testing. 2017. Antimicrobial susceptibility testing EUCAST disk diffusion method. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/Version_5/Manual_v_6.0_EUCAST_Disk_Test_final.pdf . Accessed on 01.09.2017. 7. Grundmann H., Glasner C., Albiger B., Aanensen DM, Tomlinson CT, Andrasevic AT, Canton R., Carmeli Y., Friedrich AW, Giske CG et al. 2017. Occurrence of carbapenemase-producing *Klebsiella pneumoniae* and *Escherichia coli* in the European survey of carbapenemase-producing Enterobacteriaceae (EuSCAPE): a prospective, multinational study. The Lancet Infectious diseases, 17:153-163. 8. Han R., Shi Q., Wu S., Yin D., Peng M., Dong D., Zheng Y., Guo Y., Zhang R., Hu F et al. 2020. Dissemination of Carbapenemases (KPC, NDM, OXA-48, IMP, and VIM) Among Carbapenem-Resistant *Enterobacteriaceae* Isolated From Adult and Children Patients in China. Frontiers in cellular and infection microbiology, 10:314. 9. Knezevic P., Petrovic O. 2008. Antibiotic resistance of commensal *Escherichia coli* of food-producing animals from three Vojvodinian farms, Serbia. International journal of antimicrobial agents, 31:360-363. 10. Krnjajić D., Mišić D., Ašanin R. 2005. Investigation of sensitivity and resistance to antibiotics and chemotherapeutics in *E. coli* strains isolated from animals bred in intensive farming conditions. Acta Veterinaria, 55:501-509. 11. Li X., Liu H., Cao S., Cheng P., Li F., Ishfaq M., Sun J., Zhang X. 2021. Resistance Detection and Transmission Risk Analysis of Pig-Derived Pathogenic *Escherichia coli* in East China. Frontiers in veterinary science, 8:614651. 12. Mitchaothai J., Srikijkasemwat K. 2022. Antimicrobial resistance in fecal *Escherichia coli* from different pig production systems. Animal bioscience, 35:138-146. 13. Monger XC, Gilbert AA, Saucier L., Vincent AT. 2021. Antibiotic Resistance: From Pig to Meat. Antibiotics, 10. 14. Murugan MS, Sinha DK, Vinodh Kumar OR, Yadav AK, Pruthvishree BS, Vadhana P., Nirupama KR, Bhardwaj M., Singh BR. 2019. Epidemiology of carbapenem-resistant *Escherichia coli* and first report of blaVIM carbapenemases gene in calves from India. Epidemiology and infection, 147:159. 15. Na G., Lu Z., Gao H., Zhang L., Li Q., Li R., Yang F., Huo C., Yao Z. 2018. The effect of environmental factors and migration dynamics on the prevalence of antibiotic-resistant *Escherichia coli* in estuary environments. Scientific reports, 8:1663. 16. Peng Z., Hu Z., Li Z., Zhang X., Jia C., Li T., Dai M., Tan C., Xu Z., Wu B. et al. 2022. Antimicrobial resistance and population genomics of multidrug-resistant *Escherichia coli* in pig farms in mainland China. Nature communications, 13:1116. 17. Pholwat S., Pongpan T., Chinli R., Rogawski McQuade ET, Thaipisuttikul I., Ratanakorn P., Liu J., Taniuchi M., Houpt ER, Foongladda S. 2020. Antimicrobial Resistance in Swine Fecal Specimens Across Different Farm Management Systems. Frontiers in microbiology, 11:1238. 18. Sirichokchatchawan W., Apiwatsiri P., Pupa P., Saenkankam I., Khine NO, Lekagul A., Lugsomya K., Hampson DJ, Prapasarakul N. 2021. Reducing the Risk of Transmission of Critical Antimicrobial Resistance Determinants From Contaminated Pork Products to Humans in South-East Asia. Frontiers in microbiology, 12:689015. 19. Sukalić T., Jelen T., Alagić D., Semper M., Končurat A., Pavljak I. 2019. Antimikrobna rezistencija patogenih sojeva intestinalne *Escherichia coli* u prasadi prije i poslije odbića. Veterinarska stanica, 50:315-327. 20. Xiaocao M., Z. L, Xiaohui B. 2022. Bacterial community assembly and beta-lactamase (bla) genes regulation in a full-scale chloraminated drinking water supply system. Journal of Environmental Chemical Engineering, 10:107677. 21. Yang H., Wei SH, Hobman JL, Dodd CER. 2020. Antibiotic and Metal Resistance in *Escherichia coli* Isolated from Pig Slaughterhouses in the United Kingdom. Antibiotics, 9.

TRENDS OF VETERINARY ANTIBIOTIC USE IN MONTENEGRO

Snežana Mugoša[12*], Aleksandra Kovačević[3], Dragana Tomanić[4], Zorana Kovačević[4]

[1]University of Montenegro, Faculty of Medicine, Podgorica, Montenegro
[2]Institute for medicine and medical devices of Montenegro, Podgorica, Montenegro
[3]University of Defence, Medical Faculty Military Medical Academy, Belgrade, Serbia
[4]University of Novi Sad, Faculty of Agriculture, Department of Veterinary medicine, Novi Sad, Serbia
[*]e-mail: mugosa.s@ucg.ac.me

Abstract

Antibiotic resistance is known as one of the biggest global problems in human and veterinary medicine affecting both human and animal health. In order to reduce bacterial resistance, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) provides insight into data on the use of antimicrobials in animals from 31 European countries. Unfortunately, data from Montenegro, as well as from many other developing countries, are not part of this report. Therefore, our study aimed to collect and estimate sales trends of antimicrobial in Montenegro in six year period. These sales have been monitored by the Institute for Medicines and Medical Devices based on annual reports of sales by the pharmaceutical companies and wholesalers. The evaluation of sales volumes for six consecutive years resulted in the highest share on sales volumes in 2020 with 16.37%, while the lowest share of antimicrobials were recorded in 2017 (13.16%). Veterinary antimicrobials sales surveillance is one of the main sources of information used to assess and monitor trends in antimicrobial therapy practices for different animal species but also to assess and manage the risks associated with antimicrobial resistance.

Key words: antibiotics, antimicrobial resistance, surveillance

Introduction

Antibiotic-resistant pathogens have increasingly become a major challenge in human and animal medicine (Jinks et al., 2016; Michael et al., 2014). Although inappropriate use of antibiotics in humans is the principal cause of resistance, antibiotic-resistant bacteria originating from animals contribute to the emergence and spread of these bacteria. The wide use of antimicrobial substances coupled with inappropriate antibiotic use, spurious, falsified, and counterfeit antimicrobial often

slipping into the medicine supply chain in animals contribute to the development of the AMR problem (Kluytmans, 2010).

The European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project collects information on how antimicrobial medicines are used in animals across the European Union (EU). This type of information is essential to identify possible risk factors that could lead to the development and spread of antimicrobial resistance in animals. The European Medicines Agency (EMA) started this project in September 2009, following a request from the European Commission to develop a harmonised approach for the collection and reporting of data on the use of antimicrobial agents in animals from EU and European Economic Area (EEA) Member States (EMA and ESVAC, 2015). Voluntary participation in the ESVAC project has increased from 9 to 31 reporting countries since 2010. EMA has established standardised units of measurement for reporting antimicrobial consumption in specific animal species, called the 'defined daily dose' and 'defined course dose' for animals**.** Moreover, ESVAC activity has prioritised establishing 'defined daily dose for animals' (DDDvet) and 'defined course dose for animals' (DCDvet) values for antimicrobials used in three major food-producing animal species: pigs, cattle and broilers (poultry) (ESVAC, 2015). The values are based on an assumed average daily dose (DDDvet) or treatment course dose (DCDvet) of active substance. They take account of differences in dosing, pharmaceutical form and route of administration used in the different species. Furthermore, Montenegro is not part of ESVAC, as well as Serbia. So, it is important to quantify antimicrobial use consuption in veterinary medicine at national level.

According to the Law on Medicines ("Official Gazette of Montenegro", No. 80/2020*)* the Institute for Medicines and Medical Devices has the authority to perform collecting and processing data on marketing and consumption of veterinary medicine produtcs (VMPs) in Montenegro. The report on the total value of sales for all veterinary medicines, as well as the volume of sales per packaging of individual medicines is submitted to the Institute according to the anatomical - therapeutic - chemical veterinary classification code (ATCvet) prescribed by the World Health Organization (ATCvet, 2022).

This study aimed to create quantitative evidence of animal antimicrobial usage patterns in Montenegro vs total VMPs in six year period, from 2016 to 2021.

Material and methods

Our study was based on the regulatory framework for the reporting of sales volumes of antimicrobial agents authorized as veterinary medicinal products in Montenegro which was formed by amendments of the Law on Medicines ("Official Gazette of Montenegro", No. 80/2020).

Pharmaceutical companies and wholesalers are obliged to annually report the volumes of VMPs sold to practices and clinics in Montenegro that operate a veterinary dispensary to Institute for Medicines and Medical Devices in Montenegro. For quantitative evidence of animal antimicrobial usage patterns in Montenegro vs total VMPs were used recors in six year period, from 2016 to 2021.

Results

In six years period (2016-2021) sales of antimicrobial VMPs in Montenegro remains stable with minor increase or decrease (below 5%) in overall sales. The lowest share of antimicrobial VMPs was recorded in 2017 (13,16%) and the highest in 2020 (16,37%).

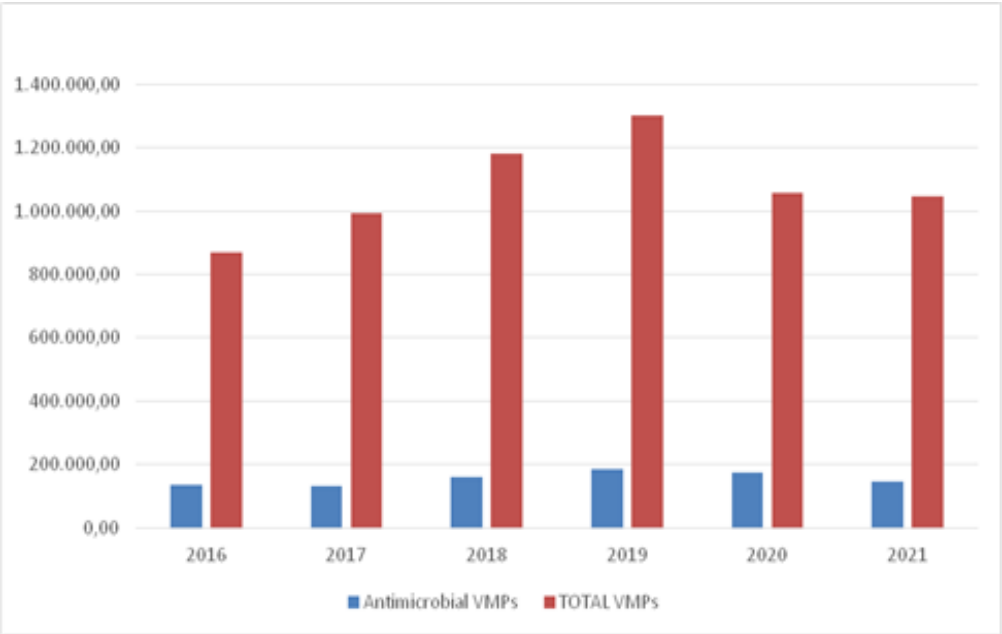


Figure 1. Sales trends (EUR) of antimicrobial vs total VMPs

Discussion

The latest ESVAC report, published in November 2021, shows that the volume of sales of antimicrobials for use in food-producing animals in Europe fell by more than 43% between 2011 and 2020. Of particular importance, sales of antimicrobials considered critically important in human medicine decreased between 2011 and 2020 by: 33% for third and fourth generation cephalosporins, 77% for polymyxins, 13% for fluoroquinolones and 85% for other quinolones. The situation across Europe remains contrasting. Out of the 25 countries that provided data covering 2011 to 2020, 19 countries observed a decline in the volume of sales of veterinary antimicrobial medicinal products of more than 5%. However, 4 countries recorded an increase of more than 5% and two other countries noted a minor increase or decrease (below 5%) in overall sales. The substantial decline in some countries indicate that there is also a potential for a decrease in other countries (ESVAC, 2021). On the other hand, our research results show stable trend in the sale of VMPs in Montenegro during the period from 2016 to 2021. Contrary to this, a considerable reduction of total sales volumes of antimicrobials for veterinary use was observed by the German surveillance system from 2011 to 2018, amounting to 983 tons (58%). Such large reductions are most likely easier to achieve in countries starting with a comparably high baseline, as it was the case in Germany. Factors affecting sales volumes are changes in animal demographics and in prescription patterns (Hauck et al., 2014; Grave et al., 2014). Changes in animal demographics are not taken into account by the German system (Dupont et al., 2016).

The World Health Organization (WHO) classifies and prioritizes antimicrobials according to their medical importance for humans (WHO, 2018). Most of the antimicrobial classes currently assigned to the category of outmost importance, the Critically Important Antimicrobials (CIA) of Highest Priority, are also authorized as VMPs. In addition, it is important to make calculation about VMPs sale regarding the class of antimicrobials, so it will be basis for our future work.

Conclusion

Research such this could serve as a baseline for surveillance of antimicrobial use and antimicrobial resistance control. A surveillance system collecting sales data at the level of pharmaceutical companies and wholesalers has to be considered an initial step to gain knowledge on antimicrobial volumes in veterinary medicine, especially regarding the retrieval of data on a global scale. To progress by identifying areas at highest risk for a targeted deduction of measures on antimicrobial use in animals and to subsequently evaluate their impact and appropriateness, a surveillance system based

on use data is required. It should be combined or even harmonized with effective monitoring programs investigating the development of antimicrobial resistance. In line with its mission, vision and objectives, Institute for Medicines and Medical Devices in Montenegro will give efforts to combat antimicrobial resistance at all levels.

Authors' contributions

SM, AK, DT, ZK: Conceptualization, writing and editing. SM: Supervision, investigation AK: Data analysis and interpretation.

Competing interests

The authors declare that they have no competing interests.

References

1. Dupont N., Fertner M., Kristensen C.S., Toft N., Stege H. 2016. Reporting the national antimicrobial consumption in Danish pigs: influence of assigned daily dosage values and population measurement. Acta Veterinaria Scandinavica, 58: 1-9. 2. European Medicines Agency, ESVAC. 2021. Sales of veterinary antimicrobial agents in 31 European countries in 2019 and 2020. Trends from 2010 to 2020. Eleventh ESVAC report. Available online: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2019-2020-trends-2010-2020-eleventh_en.pdf (accessed on 10 June, 2022) 3. European Medicines Agency, ESVAC. 2015. Vision, Strategy and Objectives 2016-2020. Available online: https://www.ema.europa.eu/en/documents/regulatory-procedural-guideline/european-surveillance-veterinary-antimicrobial-consumption-esvac-vision-strategy-2016-2020_en.pdf (accessed on 10 June, 2022). 4. European Medicines Agency. 2015. ESVAC DDDvet and DCDvet Expert Advisory. Available online: https://www.ema.europa.eu/en/documents/other/european-surveillance-veterinary-antimicrobial-consumption-esvac-defined-daily-doses-animals-dddvet_en.pdf (accessed on 10 June, 2022). 5. Grave K., Torren-Edo J., Muller A., Greko C., Moulin G., Mackay D. 2014. Variations in the sales and sales patterns of veterinary antimicrobial agents in 25 European countries. Journal of Antimicrobial Chemotherapy, 69: 2284–2291. 6. Hauck R., Römer A., Reimer I., Bender A., Haas C., Heberer T., Wallmann J. 2014. Analysis of the distribution of veterinary antimicrobial products to veterinarians in Germany in 2011 and 2012. Berliner und Munchener Tierarztliche Wochenschrift,. 127: 359–365. 7. Jinks T., Lee N., Sharland M., Rex J., Gertler N., Diver M., Jones I.., Jones K., Mathewson S., Chiara F., Farrar J. 2016. A time for action: antimicrobial resistance needs global response. Bulletin of the World Health Organization, 94: 558A–558A. 8.Kluytmans J.A.J.W. 2010. Methicillin-Resistant Staphylococcus Aureus in Food Products: Cause for Concern or Case for Complacency? Clinical Microbiology and Infection, 16:11–5. 9. Law on medicines ("Official Gazette of Montenegro", No. 80/2020). Available at: https://www.alims.gov.rs/eng/regulations/law-on-medicines-andmedical-devices/ (accessed on 10 June, 2022). 9. Michael C.A., Dominey-Howes D., Labbate M. 2014. The antimicrobial resistance crisis: causes, consequences, and management. Frontiers in Public Health, 2:145. 10. WHO Collaborating Centre for Drug Statistics Methodology. Available online: https://www.whocc.no/atcvet/ (accessed on 11 June, 2022). 11. World Health Organization. 2018. Critically Important Antimicrobials for Human Medicine, 6th revision. Ranking of medically important antimicrobials for risk management of antimicrobial resistance due to non-human use. Available online:https://apps.who.int/iris/bitstream/handle/10665/312266/9789241515528-eng.pdf (accessed on 11 June , 2022)

DIFFERENT METAPHYLACTIC APPLICATIONS ON CALVES AT RISK OF ENZOOTIC PNEUMONIA

Yigit Tan[1*], Ramazan Yildiz[2]

[1]Burdur Mehmet Akif Ersoy University, Institute of Health Sciences, Department of Internal Medicine, Burdur, Turkey

[2]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Internal Medicine, Burdur, Turkey

[*]e-mail: ramazanyildiz@mehmetakif.edu.tr

Abstract

Pneumonia is a major cause of death and economic losses to the cattle industry. Enzootic pneumonia is an important component of the bovine respiratory disease complex. Antibiotics and nonsteroidal anti-inflammatories (NSAIDs) are among the commonly used drug groups in standard treatment and prevention in calves with Enzootic Pneumonia. Metaphylaxis is defined as the group-based application of drugs, especially the widely used antimicrobials, during the pre-transplant or posttransplant arrival period for the control of pneumonia. In order to reduce antimicrobial resistance, there is a need for other drugs to be used metaphylactically to become widespread. Florfenicol, gamithromycin and tyldipyrosine are examples of some antibiotics used for metaphylactic applications. Meloxicam is a common long-acting NSAID for use in ruminants. Clinical scoring systems and monitoring systems such as body weight gain (CAA) were used. The animal material of the study was composed of 160 male Holstein calves aged 2-6 months. A daily pneumonia scoring system was used to measure the clinical disease severity. Animals were divided into 4 groups according to metaphylactic applications on the first day of arrival after transplantation. Groups were determined as Meloxicam (G1), Meloxicam plus Gamithromycin (G2), Meloxicam plus Tildiprosine (G3), Meloxicam plus Florphenicol (G4). Pneumonia scoring and flock observations were made on days 0, 3, 6 and 9. When the between group total score parameter was evaluated in the study, it was observed that there was no statistical difference between the groups on day 0. However, it was determined that the G1 and G2 groups had the lowest total score statistically ($p < 0.05$) on days 3 and 6. on the ninth day, it was determined that the G3 group had the highest total score statistically ($p < 0.05$), but there was no statistical difference between the other groups. In the study, when the body weight gains of calves were compared, it was found that statistically ($p < 0.05$) the increase in body

weight was the highest in the G1 and G2 groups, and the G3 group had the lowest value. In the study, when the body weight gains of calves were compared, it was found that statistically ($p < 0.05$) the increase in body weight was the highest in the G1 and G2 groups, and the G3 group had the lowest value. As a result, it was determined that using long-acting non-steroids similar to meloxicam instead of metaphylactic antibiotic application may be beneficial, and these and similar applications have the potential to reduce antibiotic use.
Key words: calves, enzootic pneumonia, metaphylaxis

Introductıon

Enzootic pneumonia of calves refers to infectious respiratory disease in calves. Also viral pneumonia of calves is sometimes used. Enzootic pneumonia is primarily a problem in calves <6 mounth old up to 1 year of age (Tan and Yıldız, 2020; Radostits et al., 2017). Enzootic pneumonia; presents as fever, runny nose, drooling, cough, increased respiratory rate, depression, hair loss, weak weight gain signs, and calves with partial anorexia, manifests itself as calves. Auscultation reveals abnormal lung sounds, including increased bronchial sounds, crackles, and wheezing in advanced cases. Cranial tracheal compression can cause a cough response. Advanced cases of enzootic pneumonia may show dyspnea and weakness. Virtala et al. (1996) found that fever is often the earliest manifestation of enzootic pneumonia, and this initial fever is followed by acceptable symptoms to the respiratory tract. Early manifestations of enzootic pneumonia remain subclinical to many animal handlers and must be considered pre-existing when detected by the caregiver. Pneumonia is a major cause of death and economic losses to the cattle industry. The National Animal Health Monitoring System (NAHMS) reported that approximately 21.2% (2.29 million) of beef cattle are affected by the respiratory disease (USDA–APHIS 2013). It has been reported that approximately 45-55% of all deaths in feedlot are caused by BRD (Speer et al., 2001). Bovine Respiratory Disease Complex includes environmental and management stresses, viral infection followed by a secondary bacterial infection. This is often due to environmental and management factors such as stress, poor ventilation, grouping at different ages, overcrowding, and nutritional factors such as poor quality milk replacers. Passive transfer failure is an important host factor for the development of the disease in calves. Any of several viruses may be involved and a variety of bacteria may be isolated from affected calves. Bacterial agents, including Pasteurella multocida, Mannheimia haemolytica, and Mycoplasma bovis, represent the most frequently isolated pathogenic organisms. Individual viral and bacterial etiologies, clinical manifestations, lesions, and treatment are discussed under the heading viral

respiratory ınfections in cattle and bacterial pneumonia in bovines. Accurate diagnosis and interpretation of the lesions are important in order to define the lesions developing due to pneumonia and to understand the pathogenesis of pneumonia types. Bacterial pneumonias consist of bronchopneumonia, fibrinous pneumonia and pleuropneumonia, caseonecrotic and aspiration pneumonias (Tan and Yıldız, 2020; Radostits et al., 2017). Reducing the stress factors that contribute to the development of this disease should be at the forefront in the prevention of pneumonia. Cattle groupings should be prevented from being crowded and the same age groups should be together. Mixing cattle from different regions should be avoided if possible. Transport time should be minimized and rest periods with access to feed and water should be provided during extended transport. Calves should ideally be weaned 2-3 weeks prior to shipment and dehorning and similar surgical procedures should be performed prior to transport. Cattle must be processed within 48 hours of arrival at the feedlot. Adaptation to high-energy diets should be gradual, as acidosis, indigestion, and anorexia can inhibit the immune response. Vitamin and mineral deficiencies should be corrected. Dust control measures should be used in the environment and in feed (Tan and Yıldız, 2020; Radostits et al., 2017; Urban-Chmiel and Grooms, 2012).

The different terms define "mass medication" applications in food animals, such as prophylaxis, prevention, control, group therapy, and metaphylaxis. Prophylaxis and prevention are similarly defined by the administration of a veterinary medicinal product (VMP) to healthy animals to prevent infections based on a risk or possible consequences (ECDC / EFSA / EMA 2015; EMA 2016). Typically, risk is neither clearly defined, standardized, nor measured. Initially, metaphylaxis, sometimes referred to as control or group drugs, was described as similar to prophylaxis; the difference between them is that prophylaxis is administered to individuals and metaphylaxis covers entire groups or flocks (Urban-Chmiel and Grooms 2012). In Europe, metaphylaxis is redefined as the collective drug treatment of healthy animals when the disease of interest is present in the group or herd (EMA, 2016). More precise criteria for when to administer antimicrobial metaphylaxis are rarely discussed. According to Edwards (2010) and Smith et al. (2001), the criterion for antimicrobial metaphylaxis occurs when morbidity exceeds 10% for two to three consecutive days. Other criteria used were the presence of fever in a part of the group or simply not in contact with animals showing visual clinical signs. As for the categories of animals within the major breeds produced for food, it can be summarized that the routine dosing of antimicrobials for mass medication s in calves and piglets at the neonatal stage and between 1 day and 1 week after weaning, as well as for mass medication in broiler chicks, is of great concern. A common link (e.g. weaning, feed/beef calves transport, newly formed all-in-one batch group) leading to routine antimicrobial mass medication therapy within 2–4 weeks of establishing a cohort after the stressor is identified in the majority of clinical cases. Therefore,

alternative antimicrobial products (for example, probiotics) and alternative livestock management serve an important service to prevent disease and support immunity during these transition windows of opportunistic infectious disease scenarios. An OIE symposium in 2012 identified host-derived antimicrobial peptides, prebiotics and probiotics, gene-encoded natural antibiotics including bacteriophages, recombinant synthesized enzymes such as phytases and carbohydrases, and identified five categories of potential new medical alternatives to antimicrobials, including natural phytogenic feed additives. Other alternatives include vaccination, animal welfare and biosecurity measures, animal nutrition and animal genetics measures (Seal et al., 2013). At the individual animal level, the resilience and tolerance or immune response of an animal adapting to environmental/management changes is a key factor in preventing diseases that can be cured through improved housing, biosecurity, proper nutrition, stress reduction and genetic selection vaccination (Ziping, 2018). The aim of this study is to reveal the extent to which different metaphylactic treatments applied to animals with enzootic pneumonia reduce the risk of developing pneumonia, and their clinical findings and effects. In addition to the classical applications, the differences of the applications according to different non-steroids will be revealed.

Materıals and methods

The animal material of the study was composed of 160 male Holstein calves aged 2-6 months. A daily pneumonia scoring system was used to measure the clinical disease severity. Animals were divided into 4 groups according to metaphylactic applications on the first day of arrival after transplantation. Groups were determined as Meloxicam (G1), Meloxicam plus Gamithromycin (G2), Meloxicam plus Tildiprosine (G3), Meloxicam plus Florphenicol (G4). Pneumonia scoring and flock observations were made on days 0, 3, 6 and 9.

Results

When the between group total score parameter was evaluated in the study, it was observed that there was no statistical difference between the groups on day 0. However, it was determined that the G1 and G2 groups had the lowest total score statistically ($p < 0.05$) on days 3 and 6. on the ninth day, it was determined that the G3 group had the highest total score statistically ($p < 0.05$), but there was no statistical difference between the other groups. In the study, when the body weight gains of calves were compared, it was found that statistically ($p < 0.05$) the increase in body weight was the highest in the G1 and G2 groups, and the G3 group had the lowest value. In the study, when the body weight gains of calves were compared, it was found that statistically ($p < 0.05$) the increase in body weight was the highest in the G1 and G2 groups, and the G3 group had the lowest value.

Dıscussıon and conclusion

Bovine respiratory disease (BRD) is one of the leading causes of morbidity and mortality in livestock (USDA, 2013). An estimated 16.2% of cattle placed in feeding grounds experience respiratory disease symptoms at some point during the feeding period; this rate is higher in younger and leaner cattle. 87.5% of symptomatic cattle are treated with antimicrobials, increasing antimicrobial use and costs associated with BRD. Post-weaning handling stress, intake period and pre-weaning management and nutritional status play a role in the development of this disease (Duff and Galyean, 2007).

Metaphylaxis is the group application of drugs, especially antimicrobials, which are widely used in the pre-transplant or post-transplant arrival period for the control of BRD. Metaphylaxis is an effective method to reduce the incidence of BRD in calves experiencing stress due to various factors (Duff and Galyean, 2007). Although metaphylaxis (reducing morbidity) helps achieve sustainability goals for animal welfare, it is often considered to conflict with sensible antimicrobial application goals (Word et al., 2020)

As a result, it was determined that using long-acting non-steroids similar to meloxicam instead of metaphylactic antibiotic application may be beneficial, and these and similar applications have the potential to reduce antibiotic use.

Acknowledgements

This study was supported by Burdur Mehmet Akif Ersoy University Scientific Research Project Office (Scientific Research Projects No. 0618-YL-19 and Differentiation in Agriculture and Livestock and Integrated Development Project Project No: 2017K12-41003).

Authors' contributions

YT and RY have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

1. Duff G.C., Galyean M.L. 2007. Board-invited review: Recent advances in management of highly stressed, newly received feedlot cattle. J. Anim. Sci. (85) 823-840. 2. Edwards T.A. 2010. Control methods for bovine respiratory disease for feedlot cattle. Vet Clin North Am Food Anim Pract 26:273–284. 3. EMA. 2016. European medicines agency: revised guideline for the demonstration of efficacy for veterinary medicinal products containing antimicrobial substances (EMA/CVMP/627/01-Rev.1). https://www.ema.europa.eu/en/documents/scientific-guideline/final-guideline-demonstration-efficacy-veterinarymedicinal-products-containing-antimicrobial_en.pdf. Accessed 4 June 2019. 4. Radostits O.M., Gay C.C., Hinchcliff K.W., Constable P.D. 2017.

Veterinary medicine: A textbook of the diseases of cattle, horses, sheep, pigs, goats. 11th ED. Elsevier Saunders, New York, NY. 5. Seal B.S., Lillehoj H.S., Donovan D.M., Gay C.G. 2013. Alternatives to antibiotics: a symposium on the challenges and solutions for animal production. Anim Health Res Rev. 14(1):78–87. 6. Smith RA, Stokka GL, Radostits OM, Griffin DD (2001). Health and production management in beef feedlots. Herd Health: Food Anim Prod 14:581–633. 7. Speer N.C., Young C., Roeber D. 2011. The importance of preventing bovine respiratory disease: a beef industry review. Bovine Pr. 35:189–96. 8. Tan Y., Yıldız R. 2022. Yeni sütten kesilmiş besi buzağılarında transport sonrası klinik gözlemler.. Mehmet Akif Ersoy Üniversitesi Sağlık Bilimleri Enstitüsü Dergisi. 8(3): 114-118. 9. Urban-Chmiel R., Grooms D.L. 2012. Prevention and control of bovine respiratory disease. J Livest Sci. 3:27–36. 10. USDA. 2013. Vaccine usage in feedlots. In: A. USDA, National Animal Health Monitoring System. Report 672.0153. 11. USDA–APHIS–VS. 2013. National Animal Health Monitoring System Beef Feedlot Study 2011. Part IV: Health and Health Management on U.S. Feedlots with a Capacity of 1,000 or More Head. Available from: https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_dr_PartIV.pdf. 12. Virtala AK, Mechor GD, Grohn GT (1996). Epidemiologic and pathologic characteristics of respiratory tract disease in dairy heifers during the first three months of life. J Am Vet Med Assoc. 208:2035. 13. Word AB, Wickersham TA, Trubenbach LA, Mays GB, Sawyer JE 2020. Effects of metaphylaxis on production responses and total antimicrobial use in high-risk beef calves. Applied Animal Science. (36)2, 265-270. 14. Ziping WU. 2018. Antimicrobial use in food animal production: situation analysis and contributing factors. Front Agr Sci Eng 5(3):301–311.

RESISTANCE TO BETA-LACTAMS IN PREDOMINANT STRAINS ISOLATED IN CASES OF BOVINE MASTITIS

Dragana Tomanić[1*], Zorana Kovačević[1], Ivan Stančić[1], Ivan Galić[1]

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: tomanicd@live.com*

Abstract

Antibiotics are an important component of bovine mastitis treatment and control. However, the excessive use of antibiotics in dairy herds may lead to increased drug resistance among mastitis pathogens. The emergence of drug resistance is a serious challenge for mastitis control and treatment, therefore, the present study aimed to determined prevalence of antimicrobial resistance pattern to beta-lactams in two dominant isolated strains. The present study was conducted by collecting milk samples from the cows diagnosed with clinical or subclinical mastitis and testing antimicrobial susceptibility of two predominant strains. A total of 80 milk samples were processed for bacteriology and antimicrobial susceptibility testing by the disc diffusion method. The most common isolated pathogen was *S. aureus* with 13 positive findings, while *E.coli* was isolated in 10 samples. Most of the strains were susceptible to ceftriaxone, while full resistance was observed to penicillin and cloxacillin. This study confirmed well known worldwide problem of high level of antibiotic resistance to beta-lactams among strains isolated from bovine mastitis. Our findings can assist Serbian veterinarians to choose the most appropriate antibiotic for initial empirical therapy.

Key words: antibiotics, antimicrobial resistance, beta-lactams, bovine mastitis

Sažetak

Antibiotici su važna komponenta lečenja i kontrole mastitisa krava. Međutim, prekomerna upotreba antibiotika može dovesti do povećane rezistencije na lekove među uzročnicima mastitisa, što predstavlja ozbiljan izazov za kontrolu i lečenje ovog obolenja. Stoga, ovo ispitivanje ima za cilj da utvrdi nivo antimikrobne rezistencije dva dominantna izolovana uzročnika mastitisa na beta-laktame. Uzorci mleka su prikupljeni od krava kojima je dijagnostikovan klinički ili supklinički mastitis, a potom je ispitano prisustvo antimikrobne rezistencije za dva najčešće izolovana uzročnika. Od ukupno 80 uzoraka mleka, najčešće izolovani patogen bio je *S. aureus*

sa 13 pozitivnih nalaza, dok je *E.coli* izolovana u 10 uzoraka. Većina sojeva bila je osetljiva na ceftriakson, dok je apsolutna rezistencija primećena na penicilin i kloksacilin. Rezultati ispitivanja su potvrdili dobro poznat problem visokog nivoa rezistencije uzročnika mastitisa na beta-laktame širom sveta. Dobijeni rezultati mogu pomoći veterinarima u Srbiji da odaberu najprikladniji antibiotik za inicijalnu empirijsku terapiju.
Ključne reči: antibiotici, antimikrobna rezistencija, beta-laktami, mastitis krava

Introduction

Bovine mastitis represents an important disease of dairy cattle worldwide, which can lead to serious impact on animal welfare, economy and the quality of the produced milk (Ajose et al., 2022; Hillerton and Berry, 2005). Furthermore, it has also been identified as a serious public health concern due to the presence of bacteria and their toxins in milk which enter the human food chain (Serdal and Funda, 2021). Bovine mastitis occurs in two different forms subclinical (SCM) and clinical mastitis (CM). The global prevalence rates of SCM and clinical CM are 42 and 15%, respectively (Ajose et al., 2022). Clinical form manifests with visible changes of the milk and clinical signs of infection, such as fever, redness, pain, and swelling of udder and lymph nodes (Pedersen et al., 2021). On the other hand, SCM is more common and results in reduced milk production and changes in its physical and chemical qualities, without observable clinical symptoms. Hence, it is difficult to diagnose and represents a challenge in veterinary practice (Abebe et al., 2016).

Mastitis is defined as an inflammatory response of the mammary gland caused usually by bacteria. Although more than 130 microorganisms have been reported to cause disease of which the most common bovine mastitis pathogens are commonly classified as either contagious or environmental mastitis pathogens (Pascu et al., 2022; Hillerton and Berry, 2005). Major contagious mastitis pathogens include *Staphylococcus aureus*, *Streptococcus agalactiae* and *Mycoplasma bovis* (Radinovic et al., 2021; Abdi et al., 2021). Environmental mastitis pathogens include coliforms (*Escherichia coli*, *Klebsiella*, *Enterobacter* and *Citrobacter)*, environmental streptococci (*Streptococcus uberis* and *Streptococcus dysgalactiae*) and *Corynebacterium pyogenes* (Radinovic et al., 2021; Abdi et al., 2021).

In the dairy industry, antimicrobials have been mainly used for the treatment of bovine mastitis (Boireau et al., 2018). The main treatment of mastitis is commonly administered by intramammary or parenteral administration of antibiotics, such as beta-lactams (penicillins, ampicillin, cloxacillin) aminoglycosides (streptomycin), macrolides (erythromycin), lincosamide, tetracycline, sulfonamides (Abdi et al., 2021;

Vakanjac et al., 2013).

Unfortunately, despite the best possible antimicrobial treatments, failures of bacteriological cure are frequent, especially for *S. aureus* mastitis with reported clinical cure rates as low as 4% (Breyne et al., 2017; Saini et al., 2012). Antimicrobial resistance (AMR) is considered one of the reasons for low cure rates (Barkema et al., 2006). Moreover, AMR is a major global threat of increasing concern for human, animal and environmental health. It also has implications for food safety and food security and the economic well-being of millions of farming households (Grundin et al., 2020). Monitoring AMR patterns of bacterial isolates from mastitis cases are essential for the selection of the appropriate treatment (Abdi et al., 2021). Consequently, pathogen isolation and antibiogram have become indispensable for a successful treatment and control (Serdal and Funda, 2021).

Therefore, the aim of our study was to estimate the AMR level to beta-lactams of the 2 main bacteria isolated from mastitis cases. Also, further objective was to make a comparasion with other findings.

Material and methods

Milk sample collection

The study was carried out on 80 milk samples collected from 73 dairy cows with clinical and subclinical mastitis in dairy farm located in Vojvodina, Serbia. Clinical mastitis was diagnosed by clinical examination, while subclinical form was diagnosed by analysis of milk samples. Milk samples were collected from individual quarters with mastitis to sterile tubes under asepsis and antisepsis conditions. Each teat was dipped into antiseptic solution, dried with an individual paper towel, and the teat top was scrubbed with 70% alcohol. Milk sample from each quarter was collected after the first 2–3 squirts were discarded. For each sample, cow identification number and a quarter of a cow were recorded. Briefly, milk sampling procedure was performed according to Laboratory Handbook on Bovine Mastitis of the National Mastitis Council (Hogan et al., 1999). After the milk samples were collected, they were immediately transported at the Laboratory for Milk Hygiene at the Department of Veterinary Medicine, Faculty of Agriculture, University of Novi Sad under the cold chain (4°C).

Microbiological examination

Microbiological identification and isolation were performed according to the method described by Kovačević et al. (2021). The milk samples collected from infected quarters were inoculated on 2% blood agar, using a platinum loop (0.01 mL),

and they were incubated at 37°C for 48h. Besides, further biochemical tests, catalase, coagulase and oxidase tests were performed for more specific identification.

Antibiogram

Bacteria isolated from milk samples were subjected to *in vitro* antibiotic sensitivity testing, using 6 antimicrobials agents by the Kirby–Bauer disc diffusion method on Mueller–Hinton agar. Commercially available antibiotic disks (Bioanalyse) were used for antibiotic susceptibility testing. The tested antimicrobials include ampicillin, penicillin, amoxicillin / clavulanic acid, ceftriaxone, amoxycillin and cloxacillin. The isolates and reference strains were inoculated on nutrient broth separately and incubated aerobically at 37°C. After overnight incubation, the bacterial suspension was vortexed and diluted to a turbidity equivalent to that of 0.5 McFarland standards. The bacterial suspension was then spread onto the surface of the Mueller–Hinton agar to make confluent growth. Finally, the antibiotic discs were placed into petri dishes under sterile conditions and were incubated aerobically at 37°C for 16h. The antimicrobial sensitivity of bacteria was examined on the basis of zone diameter and interpreted as sensitive or resistant.

Results

Isolates

Out of a total of 80 milk samples from cows diagnosed with mastitis, 41 samples (51.25%) were positive for the bacteriological cause of mastitis. The predominant pathogen isolates recovered from bovini mastitis cases in this study are *S. aureus* (16.25%) followed by *E. Coli* (12.5%). Prevalence of other minor pathogens was presented on fig. 1.

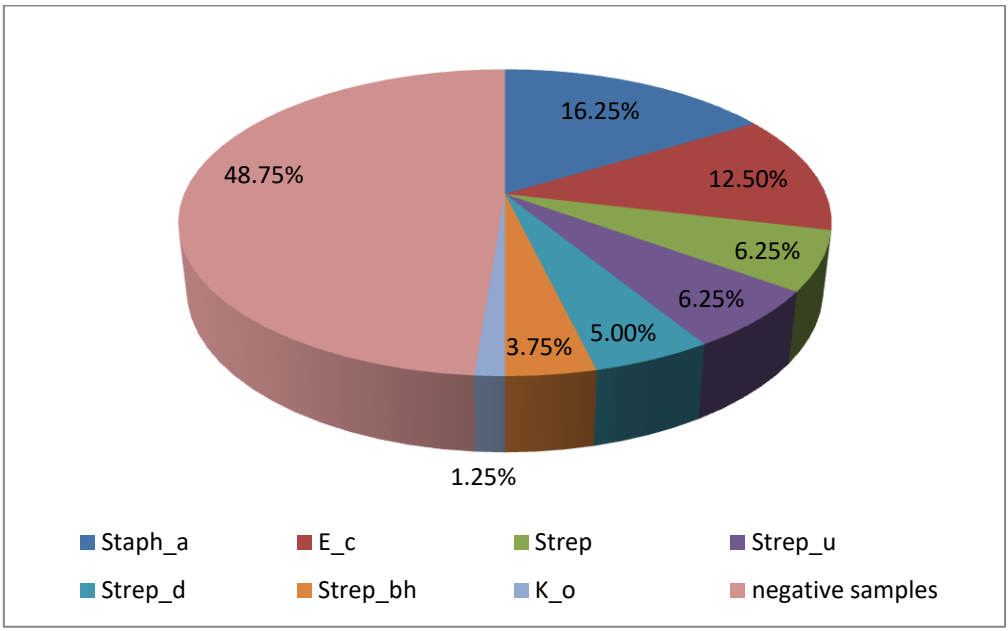


Figure 1. Proportion (%) of the evaluated bacterial strains in the collected samples. Staphylococcus aureus (Staph_a), E. coli (E_c), Streptococcus spp. (Strep), *Streptococcus uberis* (Strep_u), Streptococcus dysgalactiae (Strep_d), Streptococcus spp. β heamoliticus (Strep_bh), Klebsiella oxytoca (K_o)

Antimicrobial Resistance Patterns of isolates

Antimicrobial susceptibility patterns for the analyzed predominant mastitis-associated bacteria are shown in Table 1 and 2. Antibiotics included in the testing are amoxycillin (AMX), ampicillin (AMP), ceftriaxone (CRO), penicillin (PEN), amoxicillin/clavulanic acid (AMC) and cloxacillin (CLO). The antibiogram conducted on the bacteria isolated from infected milk samples showed different results, which may vary from agent to agent. Penicillin and cloxacillin had no effect on any bacterial isolate while all isolates showed high sensitivity to ceftriaxone. Amoxicillin/clavulanic acid had high effect only on *S. aures.* Considering all cases,

ampicillin, amoxycillin and penicillin were the least effective antimicrobial agents, while the most effective antibiotic was, ceftriaxone followed by amoxicillin/clavulanic acid.

Table 1. Antimicrobial sensitivity pattern of *S. aureus* strains isolated from cows with mastitis (S—sensitive, R—resistant).

Antibiotic	Resistant		Susceptible	
	N	%	N	%
Amoxycillin	8	61.54	5	38.46
Ampicillin	10	76.93	3	23.07
Ceftriaxone	1	7.70	12	92.30
Penicillin	13	100	0	0
Amoxicillin/clavulanic acid	2	15.38	11	84.62
Cloxacillin	13	100	0	0

Table 2. Antimicrobial sensitivity pattern of *E coli* strains isolated from cows with mastitis (S—sensitive, R—resistant).

Antibiotic	Resistant		Susceptible	
	N	%	N	%
Amoxycillin	8	80	2	20
Ampicillin	10	100	0	0
Ceftriaxone	0	0	10	100
Penicillin	10	100	0	0
Amoxicillin/clavulanic acid	6	60	4	40
Cloxacillin	10	100	0	0

Discussion

Antimicrobials are important part of mastitis therapy (Pascu et al., 2022; Vakanjac et al., 2013; Pyörälä, 2009), where more or less 60–70% of the antimicrobials used in cattle are for the prevention and treatment of mastitis (Ajose et al., 2022). However, the treatment is anticipated to become problematic due to an increase in the number of resistant strains (Ajose et al., 2022; Pascu et al., 2022). For this reason, effective treatment of bovine mastitis depends on the antimicrobial

susceptibility of the pathogens.

Determination of the prevalence of causative pathogens is highly important in order to effectively prevent diseases and to guide treatment (Bi et al., 2016). Predominant causative pathogens in our study were *S. aureus* and *E. coli* which are already described as the most frequently isolated bovine mastitis pathogens (Saidani et al., 2018; Bi et al., 2016). *S. aureus* was the most frequently isolated pathogen in our study. Such levels of *S. aureus* are comparable with those described by Bi et al. (2016) and Acharya et al. (2021). Relatively high occurrence of *S. aureus* is in agreement with literature data where this causative agent is recognized worldwide as one of the leading and most prevalent pathogens of bovini mastitis (Campos et al., 2022; Liu et al., 2020; Monistero et al., 2018; Hristov et al., 2005).

Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows (Tenhagen et al., 2006; Pitkala et al., 2004; Erskine et al., 2002). Differences in resistance patterns between reports may be caused to some extent by the variation in methods used for the determination of resistance against antimicrobial agents (Tenhagen et al., 2006). Beta-lactam group of antibiotics is widely used in therapy for bacterial infections in cattle, particularly for the treatment of mastitis (Fejzić et al., 2014; Chandrasekaran et al., 2014; Lehtolainen et al., 2003). Questionnaire based study carried out by Vidovic et al. (2022) revealed that beta-lactams are among the most popular agents used in veterinary practice in Serbia which was confirmed by Andjelkovic and Radonjic (2016).

In recent times, an increasing antimicrobial resistance rate has been recognized in *S. aureus* from bovine mastitis due to the large usage of antibiotics for the long-term (Szweda et al., 2014; Saini et al., 2012). Despite the antibiotic sensitivity that most *S. aureus* mastitis isolates display *in vitro* they are more difficult to eliminate *in vivo* compared with infections caused by other microorganisms (Breyne et al., 2017; Szweda et al., 2014). According to Cervinkova et al. (2013) high level of resistance of *S. aureus* could be explained by its capability to form biofilms, but also due to the unusually frequent acquisition of antibiotic resistance mechanisms among this group of bacteria (Szweda et al., 2014).

Resistance of *S. aureus* to penicillin or ampicillin has been extensively studied (Erskine et al., 2004), while information on the susceptibility of *S. aureus* from milk to amoxicillin/clavulanic acid is rare in the literature (Tenhagen et al., 2006). In our study, *S. aures* showed full resistance to penicillin (100%), while resistance to ampicillin and amoxicillin/clavulanic acid was expressed in lower rate. In contrary to our findings some authors (Kalmus et al., 2011; Turutoglu et al.,2006) reported lower rates of antimicrobial resistance to penicillin, ampicillin and

amoxicillin, while Klimiene et al. (2011) had very similar antimicrobial resistance to ampicillin (78.4%) in comparison to ours (76.93%), while resistance rate for amoxicillin was higher (81.3%) than rate observed in our study. A very high resistance levels to penicillin was observed in the case of *S. aureus* isolated from mastitis in China (Gao et al., 2012; Shi et al., 2010) and Ethiopia (Haftu et al., 2012). The same strains were highly sensitive to cephalosporin's activity, and the rates of sensitivity to ceftriaxone was 92.30.%. Full reistance was also observed for cloxacillin which can be related to the fact that cloxacillin is extensively used for dry cow therapy, and it is hypothesized that cloxacillin use may select for methicillin-resistant *S. aureus* (MRSA) isolates (Saini et al., 2012). High level of sensitivity was observed to amoxicillin/clavulanic acid. This information is in line with the statements of Moroni et al. (2006), who recommend the use of a combination of these two antibiotics in order to combat subclinical forms of mastitis caused by this pathogen (Moroni et al., 2006). Combining more than one synergistic antimicrobial agent may be more effective than using a single drug, and can achieve a high cure rate (Pascu et al., 2022). In fact, *S. aureus* pathogens have developed a broad spectrum of mechanisms of antibiotic resistance, which make them difficult targets even for treatment using combine therapy (Szweda et al., 2014). It was determined that all isolated strains were resistant to penicillin, since it is the one of the most prescribed antibiotic in the treatment of mastitis in our country (Radinovic et al., 2019; Anđelkovic et al., 2017; Vakanjac et al., 2013). These findings are in line with other authors (Kovacevic et al., 2022; Kovacevic et al., 2021) who also reported that the most common mastitis pathogens in dairy cows from Serbia were resistant to penicillin. Frequent contact of bacteria with a specific antibiotic can cause an increase in resistance and decrease the effect of treatment. Studies reported that over 50% of isolates that cause mastitis were resistant to either beta-lactam drugs or penicillin (Pascu et al., 2022).

E. coli is the most common Gram-negative pathogen causing clinical mastitis in dairy cattle (Pascu et al., 2022; Liu et al., 2014). Unfortunately, *E. coli* has the potential to become nonsusceptible to almost all antimicrobial agents, and the wide use of antibiotics in curing bovine mastitis has set a selective pressure driving the spread of resistance through conjugative plasmids or pathogenicity islands, with nonsusceptible *E. coli* isolates from mastitis in dairy cows being reported repeatedly (Zhang et al., 2018). The isolates showed nonsusceptibility rates against cloxacillin, penicillin, ampicillin, amoxicillin and amoxicillin/clavulanic acid with resistance rates of 100, 100, 100, 80 and 60%, respectively. Bag et al. (2021) reported high resistance against amoxicillin (94.5%) followed by ampicillin (89.5%). In contrary to our study

low resistance rate to ampicillin was observed by Fairbrothe et al. (2015) and Lui et al. (2014). In studies on *E. coli* isolates from Finland and Israel only 7 and 10% of isolates were resistant to ampicillin (Lehtolainen et al., 2003). In comparison with a French study (Boireau et al., 2018), where resistance rate for amoxicillin was 28.1%, our study reported 80%. *E. coli* has evolved a mechanism of decreasing the expression of outer membrane protein to produce drug-resistance against the beta-lactams. The extensive use of beta-lactam antibiotics in curing and preventing bovine mastitis may contribute to this phenomenon (Zhang et al., 2018). Moreover, occurrence of multidrug resistant *E. coli* in bovine mastitis is a critical public health concern due to the possibility of transmitting zoonoses and food toxin infections (Bag et al., 2021).

Conclusion

Monitoring antimicrobial resistance patterns of bacterial isolates from cases of mastitis is important for treatment decisions. The presented results indicated a considerable prevalence of antibiotic resistant strains among bovine associated pathogens. This study showed that the dominant pathogens associated with mastitis were *S. aureus* and *E. coli*. Antimicrobial resistance was highly prevalent, which emphasize the need for more prudent antibiotic use and regular antibiogram surveillance in order to prevent the emergence and spread of resistant strains. Also, estimation of resistance rates and mastitis pathogens is crucial in selecting the most effective antimicrobial agent for therapy.

Acknowledgements

This research was supported by the Science Fund of the Republic of Serbia, PROMIS, #GRANT No 6066966

Authors' contributions

DT and ZK- Conceptualization and study design, data analysis and interpretation, writing-original draft. IS and IG- Study design, data collection and writing-review and editing. All authors have read and agreed to the published version of the manuscript

Conflict of interest

The authors declare no conflict of interest.

References

1. Abdi R.D., Gillespie B.E., Ivey S., Pighetti G.M., Almeida R.A., Kerro Dego O. 2021. Antimicrobial Resistance of Major Bacterial Pathogens from Dairy Cows with High Somatic Cell Count and Clinical Mastitis. Animals, 11: 131. 2. Abebe R., Hatiya H., Abera M., Megersa B., Asmare K. 2016. Bovine mastitis: prevalence, risk factors and isolation of Staphylococcus aureus in dairy herds at Hawassa milk shed, South Ethiopia. BMC veterinary research, 12: 1-11. 3. Acharya K.R., Brankston G., Slavic D., Greer A.L. 2021. Spatio-Temporal Variation in the Prevalence of Major Mastitis Pathogens Isolated From Bovine Milk Samples Between 2008 and 2017 in Ontario, Canada. Frontiers in Veterinary Science, 8:742696 4. Ajose D.J., Oluwarinde B.O., Abolarinwa T.O., Montso K.P., Fri J., Aremu A.O., Fayemi O.E., Ateba C.N. 2022. Combating Bovine Mastitis in the Dairy sector in an era of Antimicrobial Resistance: Ethnoveterinary medicinal option as a viable alternative approach. Frontiers in Veterinary Science, 9: 800322. 5. Anđelković J., Radonjić V. 2017. Usage of intramammary antimicrobial veterinary medicinal products in the Republic of Serbia from 2011 to 2014. Serbian Journal of Experimental and Clinical Research, 18: 27-31. 6. Bag M.A.S., Khan M.S.R., Sami M.D.H., Begum F., Islam M.S., Rahman M.M., Hassan J. 2021. Virulence determinants and antimicrobial resistance of E. coli isolated from bovine clinical mastitis in some selected dairy farms of Bangladesh. Saudi journal of biological sciences, 28: 6317-6323. 7. Barkema H.W., Schukken YH., Zadoks R.N. 2006. Invited review: The role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. Journal of dairy science, 89: 1877-1895. 8. Bi Y., Wang Y.J., Qin Y., Guix Vallverdú R., Maldonado García J., Sun W., Li S., Cao Z. 2016. Prevalence of bovine mastitis pathogens in bulk tank milk in China. PLoS One, 11: e0155621. 9. Boireau C., Cazeau G., Jarrige N., Calavas D., Madec J.Y., Leblond A., Haenni M., Gay É. 2018. Antimicrobial resistance in bacteria isolated from mastitis in dairy cattle in France, 2006–2016. Journal of dairy science, 101: 9451-9462. 10. Breyne K., Honaker R.W., Hobbs Z., Richter M., Żaczek M., Spangler T., Steenbrugge J., Lu R., Kinkhabwala A., Marchon B., Meyer E. 2017. Efficacy and safety of a bovine-associated Staphylococcus aureus phage cocktail in a murine model of mastitis. Frontiers in microbiology, 8: 2348. 11. Campos B., Pickering A.C., Rocha L.S., Aguilar A.P., Fabres-Klein M.H., de Oliveira Mendes T.A., Fitzgerald J.R., de Oliveira B.R.A. 2022. Diversity and pathogenesis of Staphylococcus aureus from bovine mastitis: current understanding and future perspectives. BMC Veterinary Research, 18: 1-16. 12. Cervinkova D., Vlkova H., Borodacova I., Makovcova J., Babak V., Lorencova A., Vrtkova I., Marosevic D., Jaglic Z. 2013. Prevalence of mastitis pathogens in milk from clinically healthy cows. Veterinarni Medicina, 58: 567–75 13. Chandrasekaran D., Venkatesan P., Tirumurugaan K.G., Nambi A.P., Thirunavukkarasu P.S., Kumanan K., Vairamuthu S., Ramesh S. 2014. Pattern of antibiotic resistant mastitis in dairy cows. Veterinary World, 7: 389-394 14. Erskine R.J., Walker R.D., Bolin C.A., Bartlett P.C., White D.G. 2002. Trends in antibacterial susceptibility of mastitis pathogens during a seven-year period. Journal of Dairy Science, 85: 1111-1118. 15. Fairbrother J.H., Dufour S., Fairbrother J.M., Francoz D., Nadeau É., Messier S. 2015. Characterization of persistent and transient Escherichia coli isolates recovered from clinical mastitis episodes in dairy cows. Veterinary microbiology, 176: 126-133. 16. Fejzić N., Begagić M., Šerić-Haračić S., Smajlović M. 2014. Beta lactam antibiotics residues in cow's milk: comparison of efficacy of three screening tests used in Bosnia and Herzegovina. Bosnian Journal of Basic Medical Sciences, 14: 155. 17. Gao J., Ferreri M., Yu F., Liu X., Chen L., Su J., Han B. 2012. Molecular types and antibiotic resistance of Staphylococcus aureus isolates from bovine mastitis in a single herd in China. Veterinary Journal, 192: 550–552. 18. Grundin J., Blanco Penedo I., Fall N., Sternberg Lewerin S. 2020. The Swedish experience –a summary on the Swedish efforts towards a low and prudent use of antibiotics in animal production. Available online: https://pub.epsilon.slu.se/17781/1/grundin_j_et_al_201012.pdf (accessed on June 7, 2022). 19. Haftu R., Taddele H., Gugsa G., Kalayou S. 2012. Prevalence, bacterial causes, and antimicrobial susceptibility profile of mastitis isolates from cows in large-scale dairy farms of Northern Ethiopia. Tropical Animal Health and Production, 44: 1765–1771. 20. Hillerton J.E., Berry E.A. 2005 Treating mastitis in the cow – a tradition or an archaism. Journal of Applied Microbiology, 98: 1250–1255. 21. Hogan J.S. 1999. Laboratory handbook on bovine mastitis. Madison, WI: National Mastitis Council. 22. Hristov S., Stanković B.M., Relić R. 2005. Clinical and subclinical mastitis in cows. Biotechnology in Animal Husbandry, 21: 29-39. 23. Kalmus P., Aasmäe B., Kärssin A., Orro T., Kask K. 2011. Udder pathogens and their resistance to antimicrobial agents in dairy cows in Estonia. Acta Veterinaria Scandinavica, 53: 4. 24. Kovačević Z., Radinović M., Čabarkapa I., Kladar N., Božin B. 2021. Natural agents against bovine mastitis pathogens. Antibiotics, 10: 205. 25. Kovačević Z., Kladar N., Čabarkapa I., Radinović M., Maletić M., Erdeljan M., Božin B. 2022. New perspective of Origanum vulgare L. and Satureja montana L. essential oils as bovine mastitis treatment alternatives. Antibiotics, 10: 1460. 26. Klimiene I., Ruzauskas M., Spakauskas V., Matusevicius A., Mockeliūnas R., Pereckiene A., Butrimaite-Ambrozeviciene C., Virgailis M. 2011. Antimicrobial resistance patterns to beta-lactams of gram-positive cocci isolated from bovine mastitis in Lithuania. Polish journal of veterinary sciences, 14: 467–472. 27. Krasucka D., Cybulski W., Klimowicz A., Dzierżawski A. 2012. Evaluation of antimicrobial agents consumption in swine and cattle in Poland based on a questionnaire in 2010. Medycyna Weterynaryjna, 68: 106–109. 28. Lehtolainen T., Shwimmer A., Shpigel N.Y., Honkanen-Buzalski T., Pyörälä S. 2003. In vitro antimicrobial susceptibility of Escherichia coli isolates from clinical bovine mastitis in Finland and Israel. Journal of Dairy Science, 86: 3927-3932 29. Liu Y., Liu G., Liu W., Liu Y., Ali T., Chen W., Yin J., Han B. 2014. Phylogenetic group, virulence factors and antimicrobial resistance of Escherichia coli associated with bovine mastitis. Research in microbiology, 165: 273-277. 30. Liu K., Tao L., Li J., Fang L., Cui L., Li J., Meng X., Zhu G., Bi C., Wang H. 2020. Characterization of Staphylococcus aureus Isolates From Cases of Clinical Bovine Mastitis on Large-Scale Chinese Dairy Farms. Frontiers in Veterinary Science, 1038 31. Monistero V., Graber H.U., Pollera C., Cremonesi P., Castiglioni B., Bottini E., Ceballos-Marquez A., Lasso-Rojas L., Kroemker V., Wente N., Petzer I.M. 2018. Staphylococcus aureus isolates from bovine mastitis in eight countries: genotypes, detection of genes encoding different toxins and other virulence genes. Toxins, 10: 247. 32. Moroni P., Pisoni G., Antonini M., Villa R., Boettcher P., Carli S. 2006. Short communication: antimicrobial drug susceptibility of Staphylococcus aureus from subclinical bovine mastitis in Italy. Journal of Dairy Science, 89: 2973-6. 33. Pascu C., Herman V., Iancu I., Costinar L. 2022. Etiology of Mastitis and Antimicrobial Resistance in Dairy Cattle Farms in the Western Part of Romania. Antibiotics, 11: 57. 34. Pedersen R.R., Krömker V., Bjarnsholt T., Dahl-Pedersen K., Buhl R., Jørgensen E. 2021. Biofilm research in bovine mastitis. Frontiers in Veterinary Science, 8: 449. 35. Persson Y., Nyman A.K.J., Grönlund-Andersson U. 2011. Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. Acta Veterinaria Scandinavica, 53: 1-8. 36. Pitkälä A., Haveri M., Pyörälä S., Myllys V., Honkanen-Buzalski T. 2004. Bovine mastitis in Finland 2001—prevalence, distribution of bacteria, and antimicrobial resistance. Journal of dairy science, 87: 2433-2441. 37. Pyörälä C. 2009. Treatment of mastitis during lactation. Irish Veterinary Journal, 62, 4: 1-5. 38. Radinovc M., Davidov I., Kovacevic Z., Stojanovic D., Galfi A., Erdeljan M. 2019. Osnovni principi terapije mastitisa krava. Veterinarski žurnal Republike Srpske, 19. 39. Radinovic M.,

Kovačević Z., Davidov I., Stanojević J. 2021. Mastitisi krava-etiologija, faktori rizika i mere kontrole. Letopis naučnih radova, 45: 113-118 40. Saidani M., Messadi L., Soudani A., Daaloul-Jedidi M., Châtre P., Ben Chehida F., Mamlouk A., Mahjoub W., Madec J.Y. Haenni M. 2018. Epidemiology, antimicrobial resistance, and extended-spectrum beta-lactamase-producing Enterobacteriaceae in clinical bovine mastitis in Tunisia. Microbial Drug Resistance, 24: 1242-1248. 41. Saini V., McClure J.T., Léger D., Keefe G.P., Scholl D.T., Morck D.W., Barkema H.W. 2012. Antimicrobial resistance profiles of common mastitis pathogens on Canadian dairy farms. Journal of dairy science, 95: 4319-4332. 42. Serdal K.U.R.T., Funda E.Ş.K.İ. 2021. Pathogen isolation and antibiogram analysis in dairy cows with clinical mastitis in Adana region, Turkey. Etlik Veteriner Mikrobiyoloji Dergisi, 32: 20-26. 43. Shi D., Hao Y., Zhang A., Wulan B., Fan X. 2010. Antimicrobial resistance of Staphylococcus aureus isolated from bovine mastitis in China. Transboundary and Emerging Diseases, 57: 221–224. 44. Suojala L., Kaartinen L., Pyörälä S. 2013. Treatment for bovine Escherichia coli mastitis–an evidence-based approach. Journal of veterinary pharmacology and therapeutics, 36: 521-531. 45. Szweda P., Schielmann M., Frankowska A., Kot B., Zalewska M. 2014. Antibiotic resistance in Staphylococcus aureus strains isolated from cows with mastitis in the eastern Poland and analysis of susceptibility of resistant strains to alternative non-antibiotic agents: lysostaphin, nisin and polymyxin B. The Journal of Veterinary Medical Science, 76: 355–62. 46. Tenhagen B.A., Köster G., Wallmann J., Heuwieser W. 2006. Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. Journal of Dairy Science, 89: 2542-2551. 47. Tomanić D., Božin B., Čabarkapa I., Kladar N., Radinović M., Maletić M., Kovačević Z. 2022. Chemical Composition, Antioxidant and Antibacterial Activity of Two Different Essential Oils Against Mastitis Associated Pathogens. Acta Veterinaria-Beograd, 72: 45-58. 48. Turutoglu H.U.L.Y.A., Ercelik S., Ozturk D. 2006. Antibiotic resistance of Staphylococcus aureus and coagulase-negative staphylococci isolated from bovine mastitis. Bulletin-Veterinary Institute In Pulawy, 50: 41. 49. Vakanjac S., Pavlović V., Magaš V., Pavlović M., Đurić M., Maletić M., Nedic S., Sočo I. 2013. Investigations of efficacy of intramammary applied antimicrobials and glucocorticosteroides in the treatment of subclinical and clinical mastitis in cows. Veterinarski glasnik, 67: 15-27. 50. Vidović J., Stojanović D., Cagnardi P., Kladar N., Horvat O., Ćirković I., Bijelić K., Stojanac N., Kovačević Z. 2022. Farm Animal Veterinarians' Knowledge and Attitudes toward Antimicrobial Resistance and Antimicrobial Use in the Republic of Serbia. Antibiotics, 11:64. 51. Zhang D., Zhang Z., Huang C., Gao X., Wang Z., Liu Y., Tian C., Hong W., Niu S., Liu M. 2018. The phylogenetic group, antimicrobial susceptibility, and virulence genes of Escherichia coli from clinical bovine mastitis. Journal of dairy science, 101: 572-580. 52. Zhang T., Niu G., Boonyayatra S., Pichpol D. 2021. Antimicrobial Resistance Profiles and Genes in Streptococcus uberis Associated With Bovine Mastitis in Thailand. Frontiers in veterinary science, 8.

ANTIBIOTIC RESISTANCE OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM SUBCLINICAL MASTITIS BOVINE MILK

Mehmet Ali Bozkurt[1], Erhan Keyvan[1*], Soner Donmez[2], Erdi Sen[1]

[1]*Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Burdur, Turkey*

[2]*Burdur Mehmet Akif Ersoy University, Bucak School of Health, Burdur, Turkey*

[*]*e-mail: erhankeyvan@mehmetakif.edu.tr*

Abstract

Mastitis is a significant disease that affects the mammary tissue of dairy animals. *Staphylococcus aureus* is one of the most common causes of subclinical mastitis in dairy cows, and it is also one of the most common reasons for antibiotic treatment The study's goals were to investigate antibiotic resistance of *S. aureus* isolated from subclinical mastitis bovine milk. In this study, 350 dairy animals were screened for subclinical mastitis by California mastitis test (CMT). *Staphylococcus aureus* was isolated from the obtained milk samples with subclinical mastitis. In the current study, the antibiotic resistance levels of the isolates were detected as resistant to ampicillin (85.29%), penicillin (82.35%), oxacillin (61.76%), cefoxitin (58.82%), erythromycin (44.11%), vancomycin (35.29%), rifampine (38.23%), (26.47%), ciprofloxacin (17.64%), and gentamicin (17.64%). High levels of antibiotic-resistant isolates have been reported in dairy animals. The development of antibiotic alternatives is an important approach in treating mastitis.

Key words: antibiotic resistance, *Staphylococcus aureus*, mastitis

Introductıon

Mastitis is a costly disease for dairy farms because of the high treatment expenses (Duarte et al., 2015). In addition, milk composition varies as a result of mammary gland dysfunction (Korhonen and Kaartinen, 1995). Mastitis is thought to cost between 61 and 97 Euros per animal. Reduced milk yield, veterinary services, diagnostic charges, medicine applications, damaged milk, poor product quality, and the danger of contracting the same or new diseases are all factors that contribute to the cost (Hogeveen et al., 2011; Halasa et al., 2007).

Staphylococcal infections induce both clinical and subclinical mastitis and contribute for the bulk of mastitis-related economic losses on dairy farms around the world (Jamali et al., 2014; Oliveira et al., 2012; Moroni et al., 2006).

Bacterial resistance to antibiotics has become a major public health issue around the world. Antibiotic resistance has developed as a result of the widespread use of antibiotics in agriculture, animal husbandry, and medicine. The widespread use of antibiotics, particularly for the treatment of animals raised for food and as growth promoters, poses a significant threat to human health (Normanno et al., 2007). Antibiotics have been used extensively in the mastitis treatment strategy over the last 40 years (Tiwari et al., 2013). The widespread use of -lactam antibiotics in dairy cows for preventive and mastitis therapy exposes milk and dairy products at risk for methicillin-resistant *Staphylococcus aureus* (MRSA) (Sawant et al., 2005). MRSA can also cause mastitis in dairy cows (Haran et al., 2012).

Materıals and methods

In this study, 350 dairy animals were screened for subclinical mastitis by California mastitis test (CMT). *S. aureus* was isolated from the obtained milk samples with subclinical mastitis. The determination of antibiotic susceptibility in isolates was analyzed by the Kirby-Bauer disc diffusion method using eleven different antibiotic discs (Hudzicki, 2009).

Results

In the current study, the antibiotic resistance levels of the isolates were detected as resistant to ampicillin (85.29%), penicillin (82.35%), oxacillin (61.76%), cefoxitin (58.82%), erythromycin (44.11%), vancomycin (35.29%), rifampine (38.23%), (26.47%), ciprofloxacin (17.64%), and gentamicin (17.64%).

Dıscussıon

In Brazil, 59 (55.1%) *S. aureus* isolates were resistant to at least one antibiotic group. In a study on the effect of subclinical mastitis on milk yield, it was reported that it caused an average of 17.2% reduction (Mungube et al., 2005). In dairy cows, MRSA can cause mastitis. Guimaraes et al. (2017) reported 48.3 % MRSA, Wang et al. (2015) 15.5 % MRSA, Jamali et al. (2014) 11.6 % MRSA in milk with mastitis. MRSA can be introduced into the food chain without affecting milk quality (Parisi et al., 2016). The success rate of antibiotic therapy employed against S. *aureus* in mastitis has been observed to be low (Gruet et al., 2001). It has been suggested that the establishment of this scenario may be influenced by a persistent biofilm-dependent resistance situation (Babra et al., 2013).

New solutions for the treatment of clinical and subclinical mastitis have been developed due to some of the side effects of antibiotic therapy. Bacteriophage therapy, which is utilized against specific bacterial agents, is one of these alternate treatment

techniques (Tiwari et al., 2013). Phages have been demonstrated to be useful as new antibacterial agents in veterinary medicine. (Kwiatek et al., 2012). Despite numerous drawbacks, bacteriophage therapy may one day be regarded a viable alternative to antibiotics (Gomes and Henriques, 2016).

Conclusıons

High levels of antibiotic-resistant isolates have been reported in dairy animals. The development of antibiotic alternatives is an important approach in treating mastitis.

Acknowledgements

This study was supported by Burdur Mehmet Akif Ersoy University Scientific Research Projects Unit. (Project Number: 0400-YL-16).
Acknowledgement of COST Action 18217 ENOVAT.

References

1. Babra C., Tiwari J. G., Pier G., Thein T. H., Sunagar R., Sundareshan S., Mukkur T. 2013. The persistence of biofilm-associated antibiotic resistance of *Staphylococcus aureus* isolated from clinical bovine mastitis cases in Australia. Folia Microbiologica, 58:469-474. 2. Duarte C. M., Freitas P. P., Bexiga R. 2015. Technological advances in bovine mastitis diagnosis: an overview. Journal of Veterinary Diagnostic Investigation, 27(6):665-672. 3. Gomes F., Henriques M. 2016. Control of bovine mastitis: old and recent therapeutic approaches. Current Microbiology, *72*(4):377-382. 4. Gruet P., Maincent P., Berthelot X., Kaltsatos V. 2001. Bovine mastitis and intramammary drug delivery: review and perspectives. Advanced Drug Delivery Reviews, 50(3):245-259. 5. Guimaraes F. F., Manzi M. P., Joaquim S. F., Richini-Pereira V. B., Langoni H. 2017. Outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA)-associated mastitis in a closed dairy herd. Journal of Dairy Science, 100(1):726-730. 6. Halasa T., Huijps K., Østerås O., Hogeveen H. 2007. Economic effects of bovine mastitis and mastitis management: A review. Veterinary Quarterly, 29(1):18-31. 7. Haran K. P., Godden S. M., Boxrud D., Jawahir S., Bender J. B., Sreevatsan, S. 2012. Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. Journal of Clinical Microbiology, 50(3):688-695. 8. Hogeveen H., Huijps K., Lam T. J. G. M. 2011. Economic aspects of mastitis: new developments. New Zealand Veterinary Journal, 59(1):16-23. 9. Hudzicki J. 2009. Kirby-Bauer disk diffusion susceptibility test protocol. http://www.asmscience.org/docserver/fulltext/education/protocol/protocol.3189.pdf?expires=1548926604&id=id&accname=guest&checksum=1B7D54B8A0A0C58CFC7205DD63C3736D. Accessed: 15.06.2022. 10. Jamali H., Radmehr B., Ismail S. 2014. Prevalence and antibiotic resistance of *Staphylococcus aureus* isolated from bovine clinical mastitis. Journal of Dairy Science, 97(4):2226-2230. 11. Korhonen H., Kaartinen L. 1995. Changes in the composition of milk induced by mastitis, In: The bovine udder and mastitis. Eds. M. Sandholm, T. Honkanen-Buzalski, L. Kaartinen, S. Pyörälä, Gummerus, Jyväskylä, Finland, pp. 76–82. 12. Kwiatek M., Parasion S., Mizak L., Gryko R., Bartoszcze M., Kocik J. 2012. Characterization of a bacteriophage, isolated from a cow with mastitis, that is lytic against *Staphylococcus aureus* strains. Archives of Virology, 157:225–234. 13. Moroni P., Pisoni G., Antonini M., Villa R., Boettcher P., Carli S. 2006. Antimicrobial drug susceptibility of *Staphylococcus aureus* from subclinical bovine mastitis in Italy. Journal of Dairy Science, 89(8):2973-2976. 14. Mungube E. O., Tenhagen B. A., Regassa F., Kyule M. N., Shiferaw Y., Kassa T., Baumann M. P. O. 2005. Reduced milk production in udder quarters with subclinical mastitis and associated economic losses in crossbred dairy cows in Ethiopia. Tropical Animal Health and Production, 37(6):503-512. 15. Normanno G., Corrente M., La Salandra G., Dambrosio A., Quaglia N. C., Parisi A., Greco G., Bellacicco A. L., Virgilio S., Celano G. V. 2007. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. International Journal of Food Microbiology, 115:290-296. 16. Oliveira L., Langoni H., Hulland C., Ruegg P. L. 2012. Minimum inhibitory concentrations of *Staphylococcus aureus* recovered from clinical and subclinical cases of bovine mastitis. Journal Of Dairy Science, 95(4):1913-1920. 17. Parisi A., Caruso M., Normanno G., Latorre L., Sottili R., Miccolupo A., Santagada G. 2016. Prevalence, antimicrobial susceptibility and molecular typing of methicillin-resistant *Staphylococcus aureus* (MRSA) in bulk tank milk from southern Italy. Food Microbiology, 58:36-42. 18. Rabello R. F., Souza C. R. V. M., Duarte R. S., Lopes R. M. M., Teixeira L. M., Castro A. C. D. 2005. Characterization of *Staphylococcus aureus* isolates recovered from bovine mastitis in Rio de Janeiro, Brazil. International Journal of Dairy Science, 88(9):3211-3219. 19. Tiwari J. G., Babra C., Tiwari H., Williams V., De Wet S., Gibson J., Mukkur T. 2013. Trends in therapeutic and prevention strategies for management of bovine mastitis: an overview. Journal of Vaccines and Vaccination, 4(1):1-11. 20. Wang D., Wang Z., Yan Z., Wu J., Ali T., Li J., Han B. 2015. Bovine mastitis *Staphylococcus aureus*: antibiotic susceptibility profile, resistance genes and molecular typing of methicillin-resistant and methicillin-sensitive strains in China. Infection, Genetics and Evolution, 31:9-16.

ANTIMICROBIAL SUSCEPTIBILITY OF *STAPHYLOCOCCUS PSEUDINTERMEDIUS* ISOLATED FROM DOGS WITH PYODERMA AND OTITIS EXTERNA

Iva Zečević[1*], Matko Perharić[1], Zrinka Štritof[1], Suzana Hađina[1], Iva Benvin[1], Ozren Smolec[2], Josipa Habuš[1]

[1]*University of Zagreb, Faculty of Veterinary Medicine, Department of Microbiology and Infectious Diseases with Clinic, Zagreb, Croatia*

[2]*University of Zagreb, Faculty of Veterinary Medicine, Clinic for Surgery, Orthopaedics and Ophthalmology, Zagreb, Croatia*

[*]*e-mail: izecevic@vef.unizg.hr*

Abstract

Staphylococcus pseudintermedius is an opportunistic pathogen associated with numerous, localized and systemic, infections in dogs. The external auditory canals and skin are the most common sites of *S. pseudintermedius* isolation. Antimicrobial resistance in obtained isolates is rising steadily for those antimicrobials that are frequently used. The high prevalence of methicillin-resistant *S. pseudintermedius* (MRSP) in such infections is of concern and calls for action. The aim of this study was to estimate the frequency of isolation of *S. pseudintermedius* in canine otitis externa or pyoderma and to determine the antimicrobial susceptibility of the isolates. From January 2021 to May 2022, clinical specimens were collected from 122 dogs with clinical signs of otitis externa or pyoderma. Bacterial growth was detected in 77 (63.1%) dogs. Among the isolated bacteria, a prevalence of *S. pseudintermedius* was 54.5% and significant resistance to antimicrobial agents were detected. Only 30.7% of isolates were susceptible to erythromycin and clindamycin, 33.3% to ampicillin, and 42.9% to doxycycline. Higher susceptibility rates were observed for amoxycillin/clavulanic acid (69.2%), gentamicin (72.5%), cefalexin (75%), marbofloxacin (76.4%), enrofloxacin (76.6%), and neomycin (76%). A total of 21.4% of the isolated *S. pseudintermedius* strains were methicillin-resistant (MRSP), with 77.7% of these isolates also described as multidrug-resistant (MDR). Due to the increased population of companion animals, these results highlight the importance of MRSP-positive dogs in public health. Implementation of effective AMR control strategies, development of standardized EU antimicrobial treatment guidelines, and national surveillance systems for MRSP in companion animals are urgently needed.

Key words: otitis externa, pyoderma, *S. pseudintermedius*, MRSP, antimicrobial susceptibility

Introduction

Staphyloccocus pseudintermedius is a part of the commensal microbiota of the skin and mucous membranes in companion animals (Bannoehr and Guardabassi, 2012) and the most common bacterial pathogen isolated from clinical canine specimen. Although primarily associated with the external auditory canal and skin infections, it may also be involved in almost all types of systemic infections. Over the past decade, an increase in methicillin-resistant *S. pseudintermedius* (MRSP) has been observed (van Dujikeren et al., 2011). Of all clinical isolates from dogs and cats, up to 20 - 47% are methicillin resistant (Beck et al., 2012). Antimicrobial overuse, hospitalization, surgery and frequent veterinary visits in general are considered risk factors for MRSP infections (Lehner et al., 2014). MRSP has made treatment much more difficult. Nowadays, less effective antimicrobials are available for treatment, as MRSP isolates are often described as multidrug-resistant (MDR) (Ruscher et al., 2010). Because of the prevailing treatment problems, zoonotic potential, and public health concerns, multidrug-resistant MRSP strains have attracted considerable attention (McCarthy et al., 2014).

The present study focuses on estimating the frequency of isolated *S. pseudintermedius* from otitis externa and pyoderma infections in dogs and on investigating the pattern of antimicrobial susceptibility.

Materials and methods

The data analyzed in this study were obtained from the archives of Bacteriology laboratory at Department of Microbiology and Infectious Diseases with Clinic, Faculty of Veterinary Medicine in Zagreb. The study included a total of 122 dogs presented at the Veterinary Teaching Hospital with inflammation of the external auditory canal (n=73) or pyoderma (n=49) between January 2021 and May 2022. Clinical canine samples were collected during routine clinical examinations and referred to the Bacteriology laboratory for further testing. A disk-diffusion method was performed, according to Clinical and Laboratory Standards Institute (CLSI) and European Society of Clinical Microbiology and Infectious Diseases (EUCAST) guidelines.

Results

Of a total of 122 dogs, bacterial growth was detected in 77 (63.1%), only one pathogen was isolated in 59.7%, whereas two or more bacterial species were isolated in 40.3% of the cases. Among the isolated bacteria, *S. pseudintermedius* had a prevalence of 54.5% (n=42). In cases of canine otitis externa five antimicrobials were chosen for the disk-diffusion method, depending on the availability of topical ear remedies, whereas for pyoderma isolates the number of antimicrobials tested was extended. The results of the sensitivity testing are shown in Table 1 and Table 2. In this study, 9 (21.4%) *S. pseudintermedius* strains isolated from skin and ear canal were resistant to oxacillin (MRSP), with 7 (77.7%) of these isolates also described as multidrug resistant (MDR). MRSP strains were isolated in 55.6% of pyoderma cases and in 44.4% of otitis externa cases. MDR isolates were more likely to be found in pyoderma (71.4%) than in otitis externa (28.6%). All MDR isolates were resistant to one of the beta-lactamase-sensitive penicillin. In addition, MDR isolates were found to be resistant to fluoroquinolones, aminoglycosides, macrolides, and tetracyclines.

Table 1. Antimicrobial susceptibility of *S. pseudintermedius* isolates obtained from cases of otitis externa, determined by disk-diffusion method

Antimicrobial agents	Number (ratio) of sensitive, intermediate sensitive and resistant isolates		
	S	I	R
Cephalexin	21/25 (84%)	-	4/25 (16%)
Enrofloxacin	18/21 (85.7%)	1/21 (4.2%)	2/21 (9.5%)
Marbofloxacin	23/27 (85.2%)	1/27 (3.7%)	3/27 (11.1%)
Gentamicin	21/27 (77.7%)	-	6/27 (22.3%)
Neomycin	21/27 (77.8%)	2/27 (7.4%)	4/27 (14.8%)

S = sensitive; I = intermediate sensitive; R = resistant

Table 2. Antimicrobial susceptibility of *S. pseudintermedius* isolates obtained from cases of pyoderma, determined by disk-diffusion method

Antimicrobial agents	Number (ratio) of sensitive, intermediate sensitive and resistant isolates		
	S	I	R
Ampicillin	3/14 (21.4%)	-	11/14 (78.6%)
Amoxycillin/clavulanic acid	9/13 (69.2%)	-	4/13 (30.8%)
Cephalexin	10/15 (66.7%)	-	5/15 (33.3%)
Clindamycin	4/12 (33.3%)	-	8/12 (66.7%)
Erythromycin	4/12 (33.3%)		8/12 (66.7%)
Doxycycline	3/7 (42.9%)	-	4/7 (57.1%)
Enrofloxacin	5/9 (55.5%)	-	4/9 (44.5%)
Marbofloxacin	3/7 (42.9%)	1/7 (14.3%)	3/7 (42.8%)
Gentamicin	8/13 (61.5%)	-	5/13 (38.5%)

S = sensitive; I = intermediate sensitive; R = resistant

Discussion

The overall rate of *S. pseudintermedius* isolated from the external auditory canal and skin, noted in this study, suggests its importance as a causative agent of canine pyoderma and otitis externa infections. The majority of the isolates obtained were resistant to ampicillin (78.6%), which is similar to the result of a study conducted in Croatia (Matanović et al., 2012) and a recent study on antimicrobial, multidrug, and methicillin resistance in *Staphylococcus* spp. isolates in the USA (Lord et al., 2022). On the other hand, the resistance rates to amoxycillin/clavulanic acid (AMC) and oxacillin were 30.8% and 21.4%, respectively; significantly higher than in

the previous study conducted in Croatia when resistance against AMC and oxacillin were only 7.5% (Matanović et al., 2012). This result is not entirely surprising, since these antimicrobial agents are commonly used not only for the treatment of pyoderma infections, but in general.

Resistance rate for tetracyclines (57.1%), observed in this study, was moderately higher to that previously determined in Croatia (37.7%) (Matanović et al., 2012) and USA (38%) (Hartmann et al., 2005), but similar to a recent USA study (47.9%) (Lord et al., 2022).

Compared to the results of previous studies (Pedersen et al., 2007; Hariharan et al., 2006; Hartmann et al., 2005) a significantly higher resistance rate was also observed to macrolides (66.7%). High macrolide resistance may be a result of the increased use of these antibiotics in treatment of dermatological patients. Our findings show that empirical treatment of canine staphylococcal infections in Croatia should be reconsidered due to the very high number of resistant isolates.

In contrast to a previous Croatian study (Matanović et al., 2012), that found relatively low resistance rates to enrofloxacin (5.7%) and gentamicin (18%), an increase in resistance was observed in our study were resistance rate to these antimicrobials were 20% and 27.5%, respectively.

Significant difference between resistance rates to different antimicrobial classes in isolates obtained from the skin or ear canal was also observed. In general, resistance to all antimicrobial classes was considerably higher in canine pyoderma cases.

Conclusion

Given the considerable resistance to various antimicrobial classes observed in this study, guided, evidence-based treatment is essential for monitoring antimicrobial susceptibility patterns of *S. pseudintermedius* isolates in dogs. Rational use and improving infection prevention should be a recommended approach. Taking responsibility for the judicious use of antibiotics will lead to the avoidance of adverse veterinary and public health consequences.

Acknowledgment

The participation in the conference “Antimicrobial resistance in veterinary medicine – current state and perspectives” is supported by COST Action 18217 European Network for Optimization of Veterinary Antimicrobial Treatment

References

1. Bannoehr, J., & Guardabassi, L. (2012). Staphylococcus pseudintermedius in the dog: taxonomy, diagnostics, ecology, epidemiology and pathogenicity. *Veterinary dermatology*, *23*(4), 253–e52. 2. van Duijkeren, E., Catry, B., Greko, C., Moreno, M. A., Pomba, M. C., Pyörälä, S., Ruzauskas, M., Sanders, P., Threlfall, E. J., Torren-Edo, J., Törneke, K., & Scientific Advisory Group on Antimicrobials (SAGAM) (2011). Review on methicillin-resistant Staphylococcus pseudintermedius. *The Journal of antimicrobial chemotherapy*, *66*(12), 2705–2714. 3. Beck, K. M., Waisglass, S. E., Dick, H. L., & Weese, J. S. (2012). Prevalence of meticillin-resistant Staphylococcus pseudintermedius (MRSP) from skin and carriage sites of dogs after treatment of their meticillin-resistant or meticillin-sensitive staphylococcal pyoderma. *Veterinary dermatology*, *23*(4), 369–e67 4. Lehner, G., Linek, M., Bond, R., Lloyd, D. H., Prenger-Berninghoff, E., Thom, N., Straube, I., Verheyen, K., & Loeffler, A. (2014). Case-control risk factor study of methicillin-resistant Staphylococcus pseudintermedius (MRSP) infection in dogs and cats in Germany. *Veterinary microbiology*, *168*(1), 154–160. 5. Ruscher, C., Lübke-Becker, A., Semmler, T., Wleklinski, C. G., Paasch, A., Soba, A., Stamm, I., Kopp, P., Wieler, L. H., & Walther, B. (2010). Widespread rapid emergence of a distinct methicillin- and multidrug-resistant Staphylococcus pseudintermedius (MRSP) genetic lineage in Europe. *Veterinary microbiology*, *144*(3-4), 340–346. 6. McCarthy, A. J., Harrison, E. M., Stanczak-Mrozek, K., Leggett, B., Waller, A., Holmes, M. A., Lloyd, D. H., Lindsay, J. A., & Loeffler, A. (2015). Genomic insights into the rapid emergence and evolution of MDR in Staphylococcus pseudintermedius. *The Journal of antimicrobial chemotherapy*, *70*(4), 997–1007. 7. Matanović, K., S. Mekić, B. Šeol (2012): Antimicrobial susceptibility of Staphylococcus pseudintermedius isolated from dogs and cats in Croatia during a six-month period. Vet. arhiv 82, 505-517. 8. Lord, J., Millis, N., Jones, R. D., Johnson, B., Kania, S. A., & Odoi, A. (2022). Patterns of antimicrobial, multidrug and methicillin resistance among Staphylococcus spp. isolated from canine specimens submitted to a diagnostic laboratory in Tennessee, USA: a descriptive study. *BMC veterinary research*, *18*(1), 91. 9. Hartmann, F. A., D. G. White, S. E. West, R. D. Walker, D. J. Deboer (2005): Molecular characterization of Staphylococcus intermedius carriage by healthy and comparison of antimicrobial susceptibility patterns to isolates from dogs with pyoderma. Vet. Microbiol. 108, 119-131. 10. Hariharan, H., M. Coles, D. Poole, L. Lund, R. Page (2006): Update on antimicrobial susceptibilities of bacterial isolates from canine and feline otitis externa. Can. Vet. J. 47(3), 253-255. 11. Pedersen, K., K. Pedersen, H. Jensen, K. Finster, V. F. Jensen, O. E. Heuer (2007): Occurrence of antimicrobial resistance in bacteria from diagnostic samples from dogs. J. Antimicrob. Chemother. 60, 775-781.

SIGNIFICANCE OF MICROBIOLOGICAL VAGINAL SWAB IN PREGNANT BITCHES

Ivan Galić[1], Dragana Tomanić[1*], Ivan Stančić[1], Jovan Spasojević[1], Jelena Apić[2], Tomislav Barna[2], Vuk Vračar[1], Tijana Kukurić[1], Sandra Nikolić[1], Mihajlo Erdeljan[1]

[1]University of Novi Sad, Faculty of Agriculture, Department of Veterinary medicine, Novi Sad, Serbia

[2]Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

[*]e-mail: tomanicd@live.com

Abstract

The majority of puerperium infections of the puppies usually occur during the passage through the external reproductive organs of the bitch. Relatively low number of antibiotics that can be used in pregnancy, but also the increasingly present antimicrobial resistance, pose serious problems to veterinarians in Serbia. The use of certain antibiotics without previously testing antibiotic susceptibility, by dog breeders is a significant factor in the growth of antibiotic resistance that we face today. This study presents the results of microbiological swabs of the vagina of pregnant bitches and antimicrobial resistance in 2022 at the University Veterinary Clinic of Novi Sad. The microbiological examination of the vaginal swab from the 23 dogs revealed 22% samples of *Stapylococcus aureus*, 18% samples of hemolytic *Escherichia coli*, 17% samples of beta-hemolytic *Streptococcus*, 13% samples of *Escherichia coli*, 13% samples of *Staphylococcus pseudintermedius*, 9% samples of *Staphylococcus* sp., 4% sample of *Proteus* sp. and 4% sample *Pasteurella* sp.

Key words: bacterial microflora, antimicrobial resistance, reproductive tract, dog

Sažetak

Najveći broj infekcija u puerperijumu šteneta javlja se prilikom prolaska istog kroz spoljašnje reproduktivne organe kuje. Sužen izbor antibiotika koji se mogu primeniti u graviditetu, ali i sve prisutnija antimikrobna rezistencija, zadaju ozbiljne probleme veterinarima u Srbiji. Primena određenih antibiotika bez prethodnih rezultata antibiograma, od strane odgajivača pasa, predstavlja uzročno-posledični problem sa kojim se danas susrećemo. U ovom radu prikazani su rezultati mikrobioloških briseva vagine gravidnih kuja i antimikrobne rezistencije u 2022. godini na univerzitetskoj veterinarskoj klinici u Novom Sadu. Mikrobiološkim

pregledom vaginalnog brisa kod 23 kuje otkriveno je 22% uzoraka *Staphilococcus aureus*, 18% uzoraka hemolitičke *Escherichie coli*, 17% uzoraka *Streptococcus beta-hemolyticus*, 13% uzoraka *Escherichie coli*, 13% uzoraka *Staphylococcus pseudintermedius*, 9% uzoraka *Staphylococcus* sp., 4% uzoraka *Proteus* sp. i 4% uzoraka *Pasteurella* sp.

Ključne reči: bakterijska mikroflora, antimikrobna rezistencija, reproduktivni trakt, pas

Introduction

Mortality of puppies in the first days of life ranges from 15-40% (Hoskins, 2001). Bacteriologic cultures of the vaginal tract and mammary secretions of the bitches around parturition have been suggested as suitable for assessing the origin of the pathogenic bacteria responsible for neonatal infections (Münnich and Lübke-Becker, 2004; Sager and Remmers, 1990). Bacteria are often found in the vaginal swabs of healthy dogs at different stages of the estrous cycle (Johnston et al., 2001; Baker and Lumsden, 2000), as well as during pregnancy. The vaginal mucosa is almost never sterile (Root Kustritz, 2006). Clinically healthy dogs, up to 60%, show no signs of vaginal infection (Root Kustritz, 2006; Olson and Mather, 1978), and in most cases associated infections with anaerobic and aerobic microorganisms (van Duijkeren, 1992; Olson and Mather, 1978), often opportunistic pathogens (Bjurström, 1992) are present.

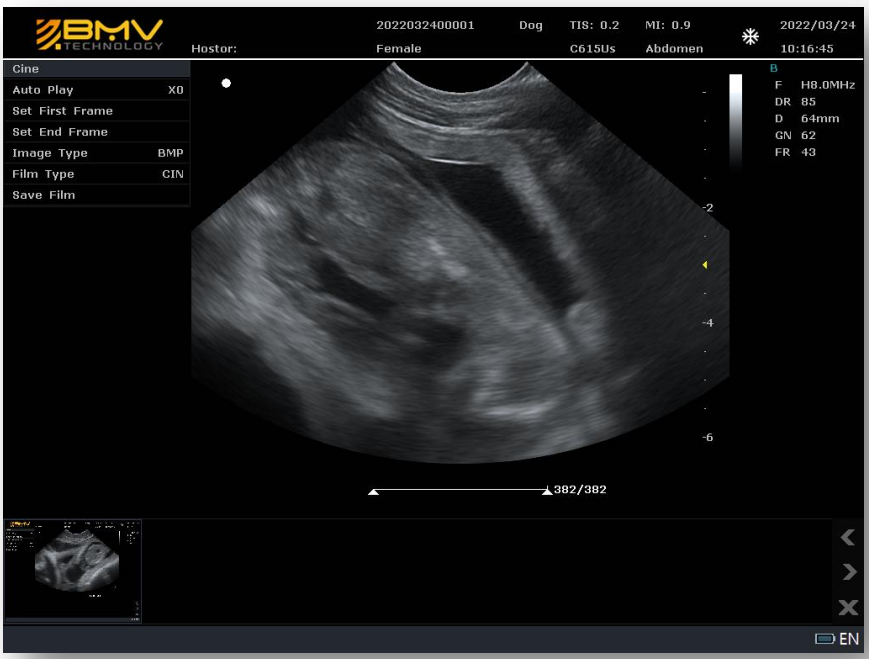
On the other hand, bacteriological isolates of bitches with reproductive disorders are often almost identical to bitches that do not have any clinical symptoms or reproductive disorder (van Duijkeren, 1992). In humans, in cases of vaginal delivery, the infant's gastrointestinal tract is colonized with vaginal and intestinal bacteria from the mother (Senn et al., 2020). Until the second half of the 19th century, it was believed that a fetus develops in a sterile environment and that the initial bacterial colonization of the newborn occurs while traveling through the maternal birth canal (Zakošek Pipan et. al., 2020). This hypothesis is also known as the sterile womb paradigm and has remained dogma for more than a century (Funkhouser and Bordenstein, 2013). However, many recent studies employing modern sequencing technologies in humans suggest that the uterus, placenta, amniotic fluid, meconium and umbilical blood from healthy pregnancies contain bacterial communities (Zakošek Pipan et. al., 2020; Stinson et al., 2019; Collado et al., 2016; Aagaard et al., 2014). This different structure of the canine placenta (endotheliochorial placenta) could minimize the transmission of bacteria from the endometrium and blood to the placenta and fetus and thereby prevent the formation of the placental microbiome and early

intrauterine microbial colonization of the puppy (Zakošek Pipan et. al., 2020). Many breeders treat bitches before and during pregnancy with different antibiotics in order to reduce the bacterial flora of the vagina and prevent neonatal mortality thus asking veterinarians to prescribe them antibiotics, ignoring antimicrobial resistance and developing opportunistic flora due to overuse of antibiotics (Rota et al., 2011; De Graef et al., 2004). Growing resistance of bacteria toward antimicrobials is becoming a concern in veterinary medicine, both for animal and human health due to the potential risk that animals may become reservoirs of zoonotic agents or that bacterial resistance may be transferred to pathogens affecting humans (Scott Weese, 2008).

Materials and methods

Pregnancy diagnosis in the bitches

Ultrasound examination was performed using an ultrasonic device BPU60 Vet (BMW, China), using a convex probe, frequency 6 MHz, in B mode. An ultrasound examination was performed between the 45th and 52nd day, and a swab was taken after the examination.



Picture 1. Pregnancy diagnosis

Vaginal swabs

Vaginal swabs were taken after cleaning the vulva with a saline solution. The labia were gently opened using sterile gloves. A sterile swab (Deltalab, Rubi, Spain) was then carefully inserted into the vagina in a craniodorsal direction and gently rotated for 15s. Use a long, guarded swab whenever possible, in order to capture the sample from the anterior vagina with as little contamination as possible.

Picture 2. Taking swabs

Bacterial isolation and antibiogram

Bacterial isolation was performed in the private veterinary laboratory VetLab using UTI agar (Urogenital tract infections chromogenic agar, HI Media, India). Then pure cultures were subcultured on the Columbia blood agar supplemented with 5% sheep blood (bioMerieux, France) using methods described by Ašanin et al. (2008). Antimicrobial susceptibility testing was performed by disk diffusion method. The standardized inoculum of the tested bacterial strain was spread on surface of Muller–Hinton agar. Antibiotic discs were placed on the surface of the agar plate and plates

were incubated at 37 ºC. After incubation, inhibition zones for bacterial growth were measured. Antimicrobial susceptibility was performed using the disk diffusion method respectively, according to the guidelines of the Institute for Clinical and Laboratory Standards and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022; CLSI, 2017).

Data analyes

The results obtained in the study were processed using Microsoft Office Excel (v2019).

Results

The microbiological examination of the vaginal swab from the 23 dogs revealed 5 (22%) samples of *Stapylococcus aureus*, 4 (18%) samples of hemolytic *Escherichia coli*, 4 (17%) samples of beta-hemolytic *Streptococcus*, 3 (13%) samples of *Escherichia coli*, 3 (13%) samples of *Staphylococcus pseudintermedius*, 2 (9%) samples of *Staphylococcus* sp., 1 (4%) sample of *Proteus* sp. and 1 (4%) sample *Pasteurella* sp. The results showing prevalence of bacterial isolates are presented in Table 1 and Figure 1.

Table 1. Frequency of isolation and microorganism species isolated from vaginal swabs.

Name of bacteria	No. of isolates
Stapylococcus aureus	5
Hemolytic *Escherichia coli*	4
Beta-hemolytic *Streptococcus*	4
Escherichia coli	3
Staphylococcus pseudintermedius	3
Staphylococcus sp.	2
Proteus sp.	1
Pasteurella sp.	1

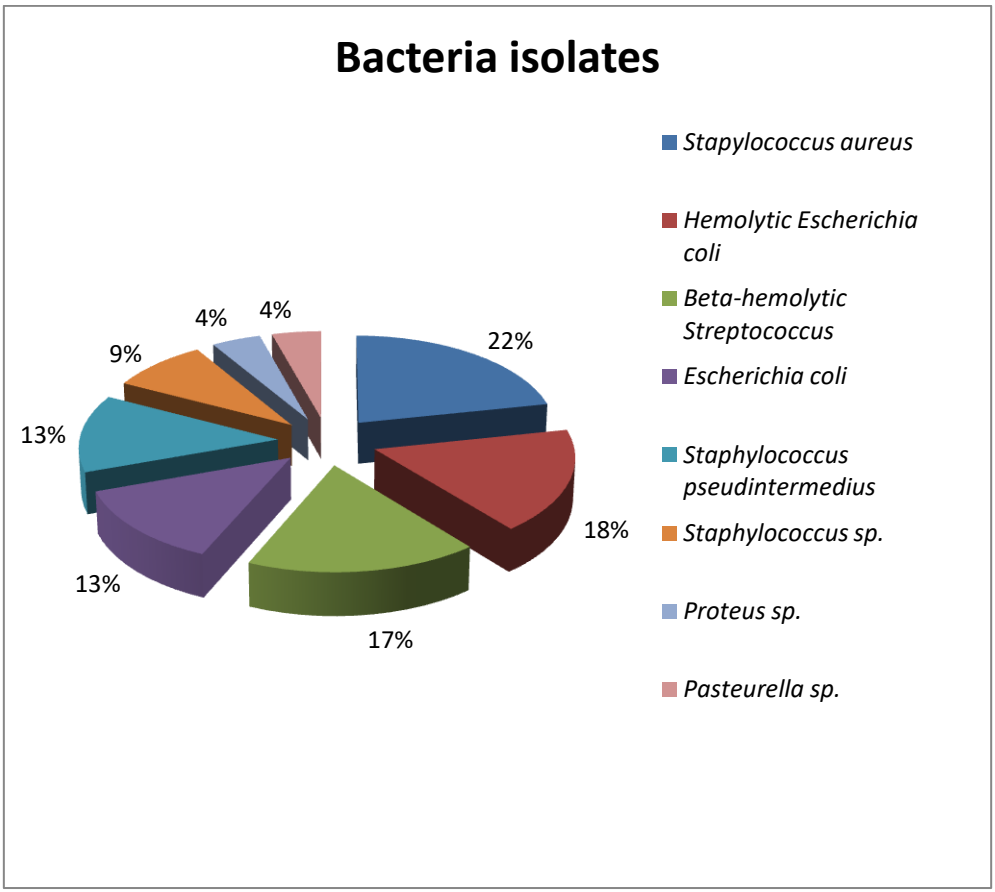


Figure 1. The proportion (%) of the evaluated bacterial strains in the collected samples.

The antibiogram of 23 vaginal swab showed that cefoxitin (78%) was the most sensitive antibiotic followed by gentamicin and ceftriaxone (74%). However, neomycin, rifampicin and enrofloxacin showed 70% sensitivity. The isolates were the most resistant to amoxiclav and amoxicillin (83%), ampicillin (74%), penicillin (70%) and doxycycline (61%).

Table 2. Overall sensitivity and resistance profiles of bacterial spp. isolated from bitches vaginal swab

Antibiotics	No. of sensitive isolates	Sensitivity %	No. of resistant isolates	Resistance %
Amoxiclav	4	17	19	83
Amikacin	11	48	12	52
Amoxicillin	4	17	19	83
Gentamicin	17	74	6	26
Neomycin	16	70	7	30
Penicillin	7	30	16	70
Trimethoprim/sulfamethoxazole	12	52	11	48
Ceftriaxone	17	74	6	26
Ciprofloxacin	15	65	8	35
Enrofloxacin	16	70	7	30
Tobramycin	10	43	13	57
Doxycycline	9	39	14	61
Azithromycin	12	52	11	48
Tetracycline	12	52	11	48
Cefalexin	15	65	8	35
Ampicillin	6	26	17	74
Vancomycin	10	43	13	57
Colistin	11	48	12	52
Pradofloxacin	13	57	10	43
Cefoxitin	18	78	5	22
Nitrofurantoin	15	65	8	35
Rifampicin	16	70	7	30
Cefquinome	14	61	9	39
Marbofloxacin	13	57	10	43

The results showing antibiotic sensitivity and resistance profiles of bacterial spp are presented in Figure 2.

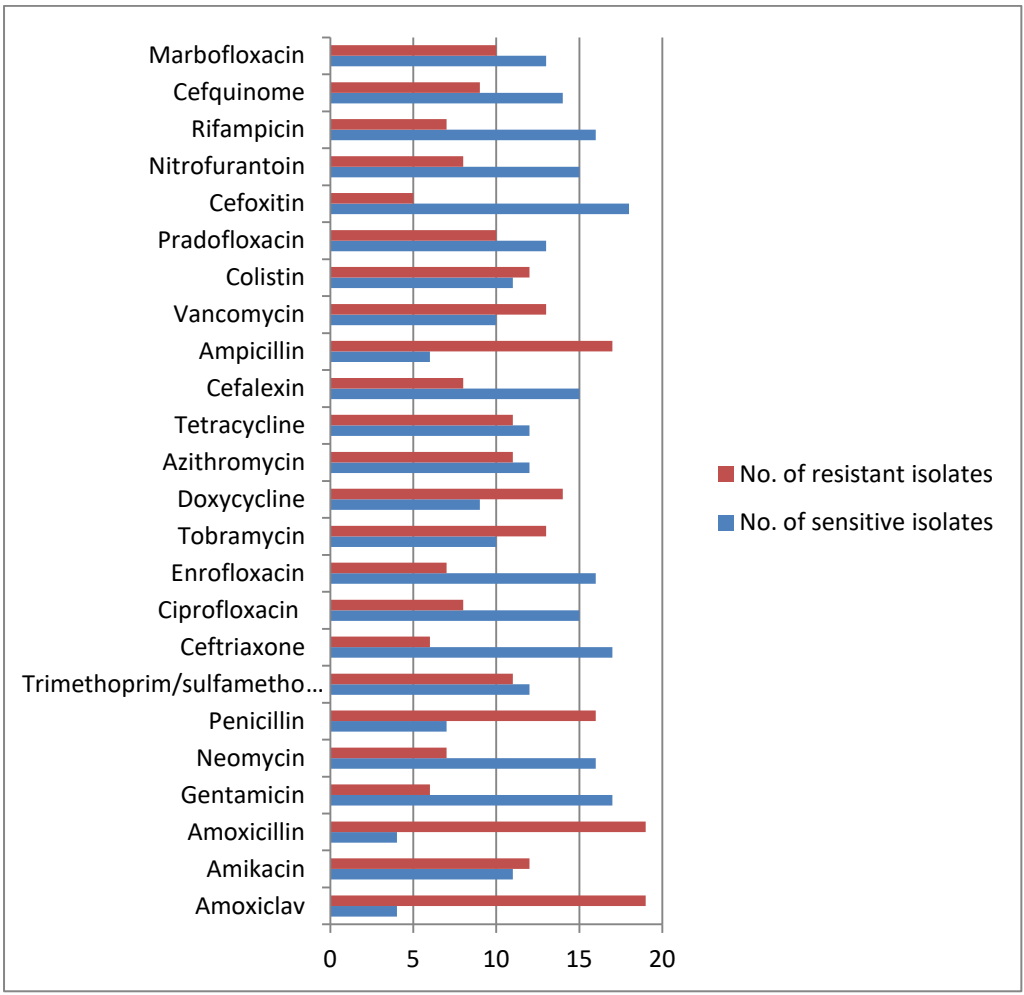


Figure 2. Antibiotic sensitivity and resistance profiles of bacterial spp.

Discussion

It is a common practice to treat bitches with antibiotics before mating, to prevent stud contamination and genital infectious diseases in females, even in the absence of clinical signs (Bjurström and Linde-Forsberg, 1992). The decision whether to treat with antibiotics is often empirical and there is little knowledge of the effects of treatment on either the bacterial flora or fertility of the bitch (van Duijkeren, 1992). There is, in fact, little information available regarding the safe use of drugs during pregnancy (Johnston et al., 2001). The canine vagina normally harbors microorganisms (Olson, 1993). Those colonizing the vaginal mucosa are usually in a

state of balance both with the host and with one another, and may therefore protect the host from pathogens (Bjurström and Linde-Forsberg, 1992). Post-mortem findings report *Escherichia coli*, *Staphylococcus* or *Streptococcus* sp. (Devriese et al., 1989) as causes of disease and death shortly after birth (Hoskins, 2001; Münnich et al., 1996). However, *Escherichia coli*, *Stapylococcus aureus*, *Staphylococcus pseudintermedius* (Rota et al., 2011; Schäfer-Somi et al., 2003), *Pseudomonas aeruginosa* or *Klebsiella* sp. are also often diagnosed in swabs taken from affected puppies or in post-mortem samples as the causative agent. Antibiotic administration affects the vaginal flora: Ström and Linde-Forsberg (1993) showed that treatment with ampicillin or trimethoprim-sulfametoxazole induces the selection of *Escherichia coli* from vaginal cultures of bitches. Our study reported that *Escherichia coli* was present in 13% of tested swabs, while resistance rate to ampicillin and trimethoprim-sulfametoxazole was 74% and 48% respectively. The normal bacterial flora plays a role in innate immunity by preventing overgrowth of bacterial species which may cause local damage or be life-threatening for puppies during colonization. Many authors have observed that organisms from healthy bitches and from ones with vaginitis are qualitatively similar (Olson, 1993; van Duijkeren, 1992) but the quantitative count is higher in bitches with vaginal discharge (Hirsh and Wiger, 1997). Saijonmaa-Koulumies and Lloyd (2002) report that the level of colonization by *Staphylococcus pseudintermedius* in bitches affects the level of colonization of their puppies, and high levels of bacterial colonization have been suggested to favour infection. The same observations apply to *Escherichia coli* (Münnich and Lübke-Becker, 2004). In comparison to our study, these two bacteria were present in 3 cases each, less than *Stapylococcus aureus*, hemolytic *Escherichia coli* and beta-hemolytic *Streptococcus*. Rota et al. (2011) showed that the bacterial flora of healthy dogs may act as a reservoir of resistance genes: a couple of *Staphylococcus pseudointermedius* strains isolated in the bitches' milk and in dead puppies in kennels with excessive use of antimicrobials were multidrug-resistant, methicillin-resistant and mecA-positive. The association between excessive antimicrobial administration to dogs and development of resistant bacterial strains has previously been shown in dogs living in kennels (De Graef et al., 2004) and in breeding kennels (Rota et al., 2011). The possibility that pets may also represent a reservoir of resistant genes and the risk of zoonotic transmission between humans and companion animals are issues under discussion (Morris et al., 2006; Meunier et al., 2004). Neonatal mortality in dogs is highest in the first week of life (Davidson, 2003) and, although it may depend on many factors, from inadequate environmental conditions to congenital malformations, genetic defects, parasitism, and infectious diseases (Hoskins, 2001), bacterial infections and septicemia are the

prevailing causes (Münnich et al., 1995). Many dog breeders believe that routine treatment of periparturient bitches with antibiotics may help to prevent neonatal morbidity and mortality (Milani et al., 2012). Due to the zoonotic potential of many organisms that may cause pregnancy loss in the queen and bitch, diligence is required to investigate any cause of pregnancy loss to determine if bacteria or protozoa may be the underlying etiology (Pretzer, 2008).

Conclusion

The use of antibiotics in pregnant bitches without previous vaginal swab testing could increase antimicrobial resistance. Majority of bacteria that are isolated from the vagina already have very high rate of resistance to most antibiotics that can be used in pregnancy. Good hygiene and veterinary practice must be undertaken in every pregnant bitch, especially in bitches kept in kennels. They must be separated from other dogs and housed in an environment that provides cleanliness and hygiene and the same applies to bitch owners. Breeders and dog owners must be aware that excessive use of antibiotics without previously conducted antibiogram does not reduce the neonatal mortality of puppies, on the contrary, it increases the risk of antibiotic-resistant bacterial strains.

References

1. Aagaard K., Ma J., Antony K.M., Ganu R., Petrosino J., Versalovic J. 2014. The placenta harbors a unique microbiome. Sci Transl Med, 6. 2. Ašanin R., Krnjajić D., Milić N. 2006. Handbook with practical exercises in microbiology, Faculty of Veterinary Medicine, Belgrade (Serbian). 3. Baker R., Lumsden J.H. 2000. Color Atlas of cytology of the dog and cat. Mosby, St. Louis. pp. 199-207. 4. Bjurström L., Linde-Forsberg C. 1992. Long-term study of aerobic bacteria of the genital tract in breeding bitches. Am J Vet Res, 53: 665-669. 5. Collado M.C., Rautava S., Aakko J., Isolauri E., Salminen S. 2016. Human gut colonisation may be initiated in utero by distinct microbial communities in the placenta and amniotic fluid. Sci Rep, 6: 23129. 6. CLSI. 2017. Performance Standards for Antimicrobial Susceptibility Testing. CLSI Supplement M100. 27th ed. Wayne, PA: Clinical and Laboratory Standards Institute. 7. Davidson A.P. 2003. Approaches to reducing neonatal mortality in dogs. Recent advances in small animal reproduction. Document A, 1226. 8. De Graef E.M., Decostere A., Devriese L.A., Haesebrouck F. 2004. Antibiotic resistance among fecal indicator bacteria from healthy individually owned and kennel dogs. Microb Drug Resist, 10: 65-69. 9. Devriese L., Ceyssens K., Hommez J., Vandermeersch R. 1989. Voorkomen van Streptococcus canis infecties bij honden, katten en runderen Vlaams. Diergeneeskd Tijdschr 58: 11-13. 10. European Committee on Antimicrobial Susceptibility Testing. 2022. Clinical breakpoint tables for interpretation of MICs and zone diameters. Version 12.0. Available online: https://www.eucast.org/clinical_breakpoints/ (accessed on June 13, 2022). 11. Funkhouser L.J., Bordenstein S.R. 2013. Mom knows best: the universality of maternal microbial transmission. PLoS Biol, 11. 12. Hirsh D.C., Wiger N. 1997. The bacterial flora of the normal canine vagina compared with that of vaginal exudates. J Small Anim Pract. 18: 25-30. 13. Hoskins J.D. 2001. Puppy and kitten losses. J.D. Hoskins (Ed.), Veterinary Pediatrics Dogs and Cats from Birth to Six Months (Third Edition), W.B. Saunders. pp. 57-61. 14. Johnston S.D., Root Kustritz M.V., Olson P.N.S. 2011. Canine and feline theriogenolog. W. B. Saunders, 1 st. ed. Philadelphia, Pa., London. 15. Meunier D., Acar J.F., Martel J.L., Kroemer S., Valle M. 2004. A sevenyear survey to marbofloxacin of pathogenic strains isolated from pets. J Int Antimicrob Agents. 24: 592-598. 16. Milani, C., Corrò, M., Drigo, M., & Rota, A. 2012. Antimicrobial resistance in bacteria from breeding dogs housed in kennels with differing neonatal mortality and use of antibiotics. Theriogenology, 78(6), 1321-1328. 17. Morris D.O., Rook K.A., Shofer F.S., Rankin S.C. 2006. Screening of Staphylococcus aureus, Staphylococcus intermedius, and Staphylococcus schleiferi isolates obtained from small companion animals for antimicrobial resistance: a retrospective review of 749 isolates (2003-04). Vet Dermatol. 17: 332-337. 18. Münnich A., Grüßel T., Leopold T.H. 1995. Experiences in diagnosis and therapy of puppy diseases in the fist days of life. Praxis. 23: 497-501.103 19. Münnich A., Grüßel T., Oelzner J., 1996: Diseases in newborn puppies - the influence of parturition and maternal health. In: 3rd Int Symposium on Reprod of Dogs, Cats and Exotic Carnivores, 69. 20. Münnich A., Lübke-Becker A. 2004. Escherichia coli infections in newborn puppies--clinical and epidemiological investigations. Theriogenology, 62: 562-575. 21. Olson P.N.S., Mather E.C.1978. Canine vaginal and uterine bacterial flora. J Am Vet Med Assoc, 172: 708-711. 22. Pretzer S.D. 2008. Bacterial and protozoal causes of pregnancy loss in the bitch and queen. Theriogenology, 70(3): 320-326. 23. Root Kustritz M.V. 2006. Collection of tissue and culture samples from the canine reproductive tract. Theriogenology. 66: 567-574. 24. Rota A., Milani C., Drigo I., Drigo M., Corrò M. 2011. Isolation of methicillin-resistant Staphylococcus pseudintermedius from breeding dogs. Theriogenology.

75: 115-121 25. Sager M., Remmers C. 1990. Perinatal mortality in dogs: Clinical, bacteriological and pathological studies. Tierarztl Prax, 18: 415-419. 26. Saijonmaa-Koulumies L.E., Lloyd D.H. 2002. Colonization of neonatal puppies by Staphylococcus intermedius. Vet Dermatol. 13: 123-130. 27. Schäfer-Somi S., Spergser J., Breitenfellner J., Aurich J.E. 2003. Bacteriological status of canine milk and septicaemia in neonatal puppies. A retrospective study. J Vet Med B Infect Dis Vet Public Health. 50: 343-346. 28. Scott Weese J. 2008. Antimicrobial resistance in companion animals. Anim Health Res Rev, 9: 169-176. 29. Senn V., Bassler D., Choudhury R., Scholkmann F., Righini-Grunder F., Vuille-Dit-Bile R.N., Restin, T. 2020. Microbial Colonization From the Fetus to Early Childhood A Comprehensive Review. Front Cell Infect Microbiol. 10: 573735. 30. Stinson L.F., Boyce M.C., Payne M.S., Keelan J.A. 2019. The not-so-sterile womb: evidence that the human fetus is exposed to bacteria prior to birth. Front Microbiol, 10: 1124. 31. Ström B., Linde-Forsberg C. 1993. Effects of ampicillin and trimethoprim-sulfamethoxazole on the vaginal bacterial flora of bitches. Am J Vet Res. 54: 891-896. 32. van Duijkeren E. 1992. Significance of the vaginal bacterial flora in the bitch: a review. Vet Rec. 131: 367-369. 33. Zakošek Pipan M., Kajdič L., Kalin A., Plavec T., Zdovc I. 2020. Do newborn puppies have their own microbiota at birth? Influence of type of birth on newborn puppy microbiota. Theriogenology. 152: 18-28.

SERBIAN MEDICAL STUDENTS' KNOWLEDGE, ATTITUDES AND BEHAVIOUR TOWARDS ANTIBIOTIC USE AND RESISTANCE: ARE THERE OPPORTUNITIES FOR IMPROVEMENT?

Bojana Jovančević[1*], Ana Tomas[1], Marija Paut Kusturica[1], Olga Horvat[1]

[1]University of Novi Sad, Faculty of Medicine, Department of Pharmacology Toxicology and Clinical Pharmacology, Novi Sad, Serbia

[*]e-mail: bokica321@gmail.com

Abstract

This study has been aimed at evaluating knowledge, attitudes and behaviour of medical students regarding the usage of antibiotics. This cross-sectional questionnaire-based study was performed on 200 final year students in 2018/2019 academic year of the integrated academic study program of medicine at the University of Novi Sad. Two thirds of the total surveyed students were female. The percentage of correct answers was higher than 85% in 9 of the 12 proposed claims related to knowledge on antbiotics, but only 68% of students gave the correct answer for the statement "Antibiotics are taken until the bottle is finished". Irregular use of antibiotics prescribed by a doctor was claimed by 21% of medical students. When it comes to taking antibiotics on their own, 37,5% of the surveyed reported they did so. The most common reason for self-medication with antibiotics was sore throat (28%) among medical students. Although a high number of students showed adequate knowledge about antibiotics, numerous misconceptions were recorded, including self-medication. Further educational interventions are necessary to improve their understanding, perceptions as well as their behaviour towards antibiotic use.

Key words: self-medication, antibiotics, resistance, Serbia, students

Sažetak

Ova studija je imala za cilj evaluaciju znanja, stavova i ponašanja studenata medicine u vezi sa upotrebom antibiotika. Studija preseka sprovedena je na 200 studenata završnih godina studijskog programa integrisane akademske studije medicine školske 2018/2019. na Univerzitetu u Novom Sadu. Ispitanici su popunjavali anonimni upitnik. Dve trećine od ukupnog broja ispitanika bile su žene. Procenat tačnih odgovora bio je veći od 85% na 9 od 12 predloženih tvrdnji u vezi sa znanjem o antibioticima, dok je samo 68% učenika dalo tačan odgovor na tvrdnju

„Antibiotici se ne uzimaju u obzir". Neredovnu upotrebu antibiotika propisanih od strane lekara prijavilo je 21% studenata medicine. Kada je reč o uzimanju antibiotika na svoju ruku, 37,5% ispitanika javilo je ovo ponašanje. Najčešći razlog za samomedicinu antibiotikom bila je upala grla (28%) među studentima medicine. Kao što je veliki broj studenata pokazao adekvatno znanje o antibioticima, zabeležene su brojne zablude i visok procenat samomedicine. Dalje edukativne intervencije su neophodne da bi se poboljšalo njihovo razumevanje, percepcija kao i njihovo ponašanje prema upotrebi antibiotika.
Ključne reči: samomedicina, antibiotici, rezistencija, Srbija, studenti

Introduction

Serbia belongs to a group of European countries with the highest rates of resistance as well as with a high antibiotic consumption rate (WHO, 2018a, b). Despite increasing concerns with antimicrobial resistance in Serbia, the implementation of physician education programs, promotion of rational use of antibiotics and hospital-based antimicrobial stewardship programs challenging have been impeded by limited resources (Kalaba et al., 2018). In addition, based on 2015 report by the WHO Europe Antimicrobial Medicines Consumption Network presenting data on 46 countries, overall consumption of antibiotics in these countries ranged from 7.66 to 38.18 DDD per 1000 inhabitants per day. Serbia with the consumption of 31.57 DDD per 1000 inhabitants per day was surpassed in total antibiotic consumption only by Greece and Turkey, while the lowest antibiotic consumption was reported in Netherlands, Germany and Austria.

Factors influencing overuse of antibiotics include doctors' competence and experience, diagnostic uncertainties as well as patients' wrong habits and their lack of knowledge (Gualano et al., 2015; Mc Nulty et al., 2007; Eng et al., 2003). So far, the only thorough study has been conducted on a representative sample of the general population in Serbia and some areas of misconceptions and improper behaviour have been identified: respondent's uncertainty as to whether antibiotics are effective against viruses and high percentage of self-medication with antibiotics (Horvat et al., 2017).

An increasing number of research focused attention on university medical students as the prospective most important antibiotic prescibers, since their knowledge, attitudes and behaviour in relation to usage of antibiotics can greatly impact antibiotic-related issues in the future (Scaioli et al., 2015; Jamshed et al., 2014; Huangn et al., 2013).

Therefore, the objective of this study was to evaluate knowledge, attitudes and behaviour of medical students regarding the usage of antibiotics in Serbia.

Materials and methods

The study was conducted in January and February 2019 at the University of Novi Sad, one of the largest Serbian Universities. The study population comprised a total of 200 final year students in 2018/2019 academic year of the integrated academic study program of medicine.The selected group of students are prospective health care professionals who will be permitted to prescribe antibiotics in the Republic of Serbia and have an imapct on antimicrobial resistance. After signing the informed consent, the respondents filled the questionnaire. The research was approved by the Ethical Committee of the Faculty of Medicine in Novi Sad (approval number 01-39 / 290/1).

The questionnaire used in this study was based on the questionnaire of Buke et al. (2003). Necessary modifications have been made to provide correct answers to questions and claims. The content, comprehension, readability and appearance of the questionnaire were previously tested on 30 students of the University of Novi Sad who were not included in the final analyses.

The questionnaire was divided into three parts. The first part, which contained 8 questions, included demographic characteristics of respondents and some general questions (age, gender, year of study, field of study, average grade, place of residence, number of visits to a general practitioner in the past 12 months, is anyone in his family healthcare worker). The second part referred to the respondents' knowledge regarding theuse of antibiotics. This part contained 12 statements with the possibility of answering with true or false. The questions were about the conditions that require the use of antibiotics, how to start antibiotic treatment, the duration of antibiotic therapy, the meaning of the claim "take twice a day", and whether frequent and inadequate use of antibiotics can be harmful. The knowledge score was determined by giving one point for each correct answer and the maximum score was 12.

The third part, which consisted of 15 questions with the possibility of answering yes or no, as well as multiple choice of answers, refered to the attitudes and behavior of students towards antibiotics. This part was focused on the prophylactic use of antibiotics, self-medication with antibiotics, adherence of the antibitic therapy, on whose reccomendation the students take antibiotics, and how they used them during the previous infection.

To examine the indication for which respondents usually took antibiotics on their own (self-medication), they were offered an additional question to name the indication in case they had taken an antibiotic on their own. The following answers (i.e., conditions) were offered: fever, cough, sore throat, common cold, abdominal pain, infection of the skin, headache, cystitis, other infections.

Descriptive statistical analysis of the results was performed with IBMSPSS Statistics

22 (IBM Corporation, Armonk, NY, USA) software. Out of descriptive statistical methods, measures of central tendency (mean), measures of variability (standard deviation) and frequency were used.

Results

Sociodemographic and academic characteristics

Almost two thirds (62.5%) of the total surveyed students were female (Table 1). Most of the examined medical students (89.5%) had high average grade (8.00-10.00).One third of the total number ofsurveyed students claimed to live in rented apartments (33,5%), living with parents (32%) and having a family member who is healthcare worker (35%). Most of them didn't visit general practitioners in the last 12 months (54,5%).

Table 1. Socio-demographic and academic characteristics of examined medical students

		N	%
Gender	Male	75	37,5
	Female	125	62,5
Average grade	6,00-7,99	21	10,5
	8,00-10,00	179	89,5
Place of living	with parents	64	32
	in university dormitories	33	16,5
	in rented apartments	67	33,5
	in own apartment	36	18
Number of visits to GP in the last 12 months	None	109	54,5
	1-4	82	41
	5-10	7	3,5
	>10	2	1
Having a family member who is healthcare worker	Yes	70	35
	No	130	65

Students' knowledge regarding the antibiotics use

Respondents showed good knowledge regarding the use of antibiotics (Table 2). The percentage of correct answers was higher than 85% in 9 of the 12 proposed claims.The lowest percentage of correct answers was recorded for the statement "Antibiotics are taken until the bottle is finished". Of the 200 students surveyed, only 68% gave the correct answer. When it comes to statements "Antibiotic is used until the symptoms disappear" and "Taking the medicine twice a day means after waking up and before going to bed", 83,5% and 81,5% of the students surveyed gave the correct answer, respectively.

Table 2. Knowledge of students of medicine

	N	%
Antibiotics are used to decrease fever	174	87
Antibiotics are used to decrease pain	190	95
Antibiotics are used to overcome malaise and fatigue	197	98,5
Antibiotics are used for common cold	171	85,5
Antibiotic treatment begins with an antibiotic found at home in order not to waste time	180	90
Antibiotic treatment is started after a visit to doctor and with a doctor's prescription	194	97
Antibiotic treatment is started when it is advised by a pharmacist	171	85,5
Antibiotic is used until the symptoms disappear	167	83,5
Antibiotic is used until the bottle finishes	136	68
Antibiotic is used as long as the doctor prescribes	194	97
Taking the medicine twice a day means after waking up and before going to bed	136	81,5
Frequent and improper use of antibiotics is harmful and dangerous	194	97

T/F: true/false and percentages denote those who gave the correct answer

Behavior and attitudes of respondents toward antibiotics use

All students surveyed denied prophylactic use of antibiotics. Irregular use of antibiotics prescribed by a doctor was reported by 21% of medical students. When it comes to taking antibiotics on their own, 37,5% of the surveyed claimed they did so. On the other hand, 72% of the students reported using the antibiotic prescribed by the doctor and 74,5% of the students reported using antibiotics as advised by the doctorduring last infection. To the question "What do you do when you think the antibiotics you are taking are ineffective?", 72% of medical students continue to take antibiotics recommended by a doctor (Table 3).

Tabela 3. Attitudes and behavior of students of medicine

	N	%
Have you ever used antibiotics in order not to get ill?		
No	200	100
Have you ever started antibiotics on your own when you got ill?		
Yes	75	37,5
Have you ever used antibiotics prescribed by the doctor irregularly?		
Yes	42	21
What do you do when you think that antibiotic you are taking is not effective?		
I stop taking it and go to the doctor	36	18
I stop taking it and go to another doctor	8	4
I use it for the recommended period	144	72
Other	12	6
How did you use antibiotics during your last infection?		
Until the bottle is finished	38	19
Until the symptoms disappeared	13	6,5
As advised by the doctor	149	74,5
How did you get antibiotics during your last infection?		
I used the antibiotic previously used or as advised by my friends or relatives	5	2,5
I used the antibiotic previously prescribed by my doctor	17	8,5
I visitied my doctor and used the prescribed antibiotic	144	72
I asked the pharmacist and used the antibiotic recommended	8	4
I do not remember	26	13

Reasons for self-medication with antibiotics

Out of the total sample of 200 medical students, the most common reason for self-medication with antibiotics was sore throat (28%), followed by cystitis (15.5%) and cough (15%) (Figure 1).

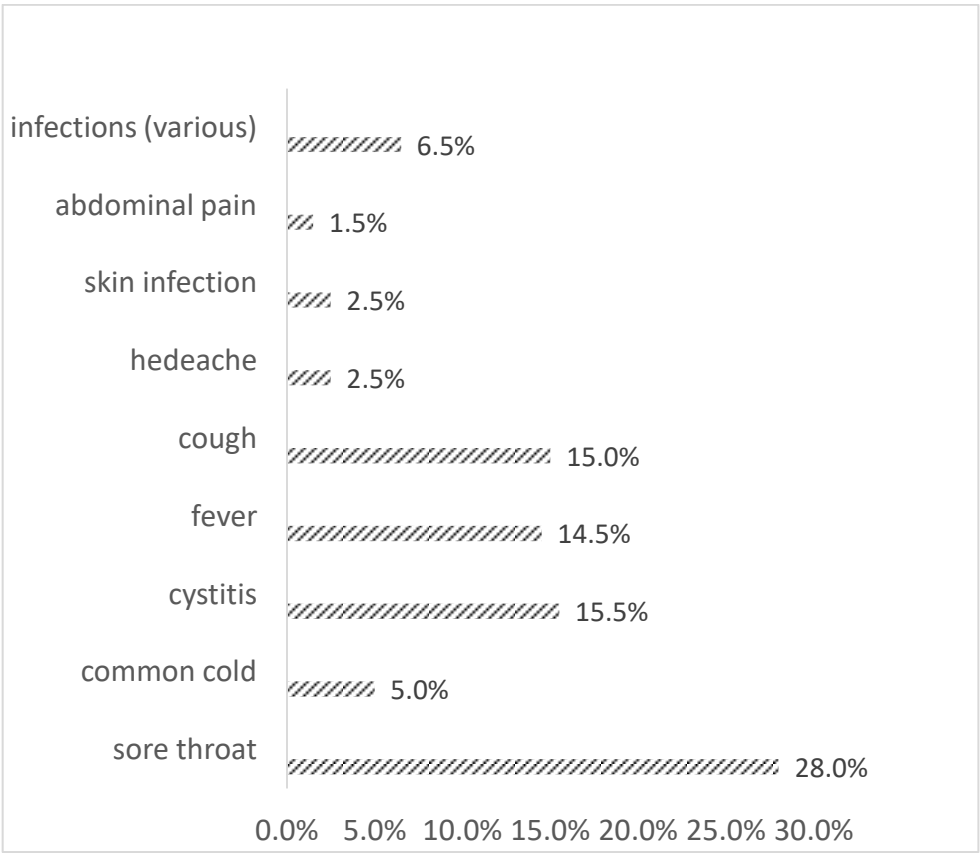


Figure 1. Reasons for self-medication with antibiotics.

Discussion

This study aimed to reflect the state of knowledge and behavior regarding antibiotic use of the Serbian medical students, as well as to elucidate factors influencing the main outcomes of interest. To the best of our knowledge, this is the first detailed study of this type conducted in Serbia. Our study showed that the interviewed students had good knowledge on antibiotics, although there were important misconceptions when the answers to the specialized question whether antibiotics can be used for the treatment of common cold were analysed (Jairoun et

al., 2019; Huangn et al., 2013; Ghadeer et al., 2012). Namely, only 85.5 % of the total sample of medical students believed that antibiotics should not be used for common cold. On the other hand, in the similar study conducted in Turkey 83.1% of the students both in medical and non medical group believed that antibiotics could be used against common cold (Buke et al., 2005). Furthermore, this result of the present study is encouraging when compared to the Serbian general population. The 2015 WHO report on Antibiotic Resistance pointed out how only 28% of the Serbian subjects interviewed believed that antibiotics were not effective against cold and flu, while 4% did not know the answer to the questiod (WHO, 2015). However, the results of the first detailed study performed on Serbian general population by Horvat et al. (2017) showed that 41.6% of the respondents stated that antibiotics were not useful for common cold. Similarly, in the first systematic review compiling 24 studies on the knowledge on antibiotic use, it was stated that about 50% of the respondents did not know that antibiotics were not effective against cold and flu (Gualano et al., 2015).

About one-third of all the students stated that antibiotics should be used until the bottle is finished. Additionally, about one-fifth of the students surveyed stated that antibiotics should be used until symptoms disappear and around the same percentage of respondents indicated that twice-daily antibiotic regimen entails taking medicine after waking up and before going to bed at night. These findings demonstrate clear misconception among our respondents regarding adherence to antibiotic regimens and are comparable with previously published data related to both medical and non medical students in India, Portugal, Italy and Turkey (Scaioli et al., 2015; Khan and Banu, 2013; Azevedo et al., 2009; Buke et al., 2005). Our results are not in agreement with the results of the study performed in USA, where almost all the medical students interviewed were aware that inappropriate use of antibiotics could harm patients and increase prevalence of resistant strains of bacteria (Minen et al., 2010; Hawkings et al., 2008).

The fact that no one reported using antibiotics for prophylactic purposes strongly supports the respondents' good knowledge regarding antibiotics. When it comes to the general population in Serbia, this percentage was 6% (Horvat et al., 2017). Around 6,5% of medical students used antibiotics only until their symptoms resolved in our study. This is similar to the results obtained in the study of medical students in India (Khan and Banu, 2013), whereas this behaviour was recorded among more than 70% of university students from Jordan (Suaifan et al., 2012), India (Limaye et al., 2018) and Portugal (Azevedo et al., 2009). This misconception in the antibiotic use may put the patients at risk of relapse with resistant pathogenic bacteria (Dyar et al., 2013; Sanya et al., 2013). However, in addition to antibiotic prescribed

by the doctor, 8.5% of our study sample students most frequently used leftover antibiotics previously prescribed by a physician. This is a considerably lower percentage than among Italian medical students (17.7%), Turkish students (25.9%) (Scaioli et al., 2015; Buke et al., 2005). In addition, it is also lower than the one reported for Serbian general population (17%) (Horvat et al., 2017). Similar to the latter study, our investigation highlights that leftovers are the most common source of self-medication in Serbia after enforcement of law restricting the purchase of antibiotics at the pharmacy without he medical prescription in 2011 (Tomas et al., 2017; Horvat et al., 2017; Kusturica et al., 2015). According to this result, Serbia is now more similar to southern, northern and western European countries where the major source of self-medication is leftover medications whereas in eastern countries purchase of antibiotics without prescription still occurs at high rate (Grigoryan et al., 2008).

Our study has shown that self-medication with antibiotics represents a common behaviour among medical students in Novi Sad (37.5%). The study performed on medical students in Italy showed that every year spent at university significantly decreased the chance of using antibiotics only when prescribed by the doctor (Scaioli et al., 2015). Similarly, the Chinese and Libyan studies documented a significantly higher rate of self-medication practice among medical students who had taken formal lectures about antibiotics (Ghaieth et al., 2015; Lv et al., 2014; Pan et al., 2012). Although it seems that students feel more confident with their knowledge regarding antibiotics use, as they attend medical related faculties, Iranian and Palestinian studies yielded the contrasting results and pointed that nonmedical students reported slightly higher rates of self-medication (Sarahroodi et al., 2010; Sawalha, 2008).

Conclusion

The results indicate that although the students have shown adequate knowledge about antibiotics, numerous misconceptions that include their belief that antibiotics are appropriate in the treatment of common cold as well as that treatment lasts until the symptoms resolve have been recorded. Additionally, our study demonstrated that dispensing whole packages of antibiotics leads to a higher number of students who are in the possession of leftover antibiotics, which is the most common source of self-medication nowadays in Serbia. Therefore, implementation of antibiotic regulation which will urge pharmacists to dispense antibiotics on unit-dose basis instead of selling a whole drug package should be taken into consideration. It seems prudent to re-evaluate the educational curricula of future antibiotic prescribers, specifically teaching of clinical pharmacology. Most importantly, there is a need to

incorporate curriculum on abuse of antibiotics and the harm of such practice on short and long run. Further research to investigate knowledge, attitudes and practice towards antibiotic in other settings will help to adopt and implement successful future educational campaigns in promotion of rational antibiotic use in Serbia where the problem of bacterial resistance is increasing at an alarming rate.

Acknowledgements

This work was supported by the Provincial Secretariat for Higher Education and Scientific Research project No. 142-451-2574/2021-01/01.

Authors' contributions

Conceptualization: OH; Data curation: OH, ADT, BJ; Formal analysis: ADT, BJ; Investigation: BJ, ADT, MPK; Methodology: OH, AT, MPK; Supervision: OH; Validation: OH, BJ, ADT, MPK; Visualization: BJ, MPK; Writing ± original draft: BJ, OH, ADT, MPK.

Competing interests

All authors declare that they have no competing interest.

References

1. Azevedo MM, Pinheiro C, Yaphe J, Baltazar F (2009) Portuguese students' knowledge of antibiotics: a cross-sectional study of secondary school and university students in Braga. BMC Public Health 9:359. https://doi.org/10.1186/1471-2458-9-359 2. Buke CA, Ermertcan S, Hosgor-Limoncu M, Ciceklioglu M, Eren S (2003) Rational antibiotic use and academic staff. Int J Antimicrob Agents 21:63–66. https://doi.org/10.1016/S0924-8579(02)00272-8 3. Buke C, Hosgor-Limoncu M, Ermertcan S, Ciceklioglu M, Tuncel M, Ko¨se T, Eren S (2005) Irrational use of antibiotics among university students. J Infect 51(2):135–139. https://doi.org/10.1016/j.jinf.2004.12.001 4. Dyar OJ, Howard P, Nathwani D, Pulcini C (2013) Knowledge, attitudes, and beliefs of French medical students about antibiotic prescribing and resistance. Me´d Maladies Infect 43(10):423–430. https://doi.org/10.1016/j.medmal.2013.07.010 5. Eng JV, Marcus R, Hadler JL et al (2003) Consumer attitudes and use of antibiotics. Emerg Infect Dis 9:1128–1135. https://doi.org/10.3201/eid0909.020591 6. Ghadeer ARY, Suaifan Mayadah Shehadeh, Darwish Dana A, Al-Ijel Hebah, Yousef Al-Motassem M, Darwish Rula M (2012) A cross-sectional study on knowledge, attitude and behavior related to antibiotic use and resistance among medical and non-medical university students in Jordan. Afr J Pharm Pharmacol 6(10):763–770. https://doi.org/10.5897/AJPP12.080 7. Ghaieth MF, Elhag SR, Hussien ME, Konozy EH (2015) Antibiotics self-medication among medical and nonmedical students at two prominent Universities in Benghazi City, Libya. J Pharm Bioallied Sci 7(2):109–115. https://doi.org/10.4103/0975-7406.154432 8. Grigoryan L, Burgerhof JG, Degener JE, Deschepper R, Lundborg CS et al (2008) Determinants of self-medication with antibiotics in Europe: the impact of beliefs, country wealth and the healthcare system. J Antimicrob Chemother 61:1172–1179. https://doi.org/10.1093/jac/dkn054 9. Gualano MR, Gili R, Scaioli G, Bert F, Siliquini R (2015) General population's knowledge and attitudes about antibiotics: a systematic review and meta-analysis. Pharmacoepidemiol Drug Saf 24(1):2–10. https://doi.org/10.1002/pds.3716 10. Hawkings NJ, Butler CC, Wood F (2008) Antibiotics in the community: a typology of user behaviours. Patient Educ Couns 73(1):146–152. https://doi.org/10.1016/j.pec.2008.05.025 11. Horvat OJ, Tomas AD, Paut Kusturica MM, Savkov AV, Bukumiric´ DU, Tomic´ ZS, Sabo AJ (2017) Is the level of knowledge a predictor of rational antibiotic use in Serbia? PLoS ONE 7:e0180799. https://doi.org/10.1371/journal.pone.0180799 12. Huangn Y, Gu J, Zhang M, Ren Z, Yang W, Chen Y et al (2013) Knowledge, attitude and practice of antibiotics: a questionnaire study among 2500 Chinese students. BMC Med Educ 13:163. https://doi.org/10.1186/1472-6920-13-163 13. Jairoun A, Hassan N, Ali A, Jairoun O, Shahwan M (2019) Knowledge, attitude and practice of antibiotic use among university students: a cross sectional study in UAE. BMC Public Health 19(1):518. https://doi.org/10.1186/s12889-019-6878-y 14. Jamshed SQ, Elkalmi R, Rajiah K, Al-Shami AK, Shamsudin SH, Siddiqui MJ et al (2014) Understanding of antibiotic use and resistance among final-year pharmacy and medical students: a pilot study. J Infect Dev Ctries 8:780–785. https://doi.org/10.3855/jidc.3833 15. Kalaba M, Kosutic J, Godman B, Radonjic V, Vujic A, Jankovic S et al (2018) Experience with developing antibiotic stewardship programs in Serbia: potential model for other Balkan countries? J Comp Eff Res 7:247–258. https://doi.org/10.2217/cer-2017-0055 16. Khan AKA, Banu GKKR (2013) Antibiotic resistance and usage-a survey on the knowledge, attitude, perceptions and practices among the medical students of a Southern Indian Teaching Hospital. J Clin Diagn Res 8:1613–1616. https://doi.org/10.7860/JCDR/2013/6290.3230 17. Kusturica MP, Tomic Z, Bukumiric Z, Horvat O, Pavlovic N, Mikov

M, Sabo A (2015) Antibiotics in Serbian households: a source of potential health and environmental threats? Cent Eur J Public Health 23(2):114–118. https://doi.org/10.21101/cejph.a4093 18. Limaye D, Naware S, Bare P, Dalvi S, Dhurve K, Sydymanov A, Limaye V, Pitani RS, Kanso Z, Fortwengel G (2018) Knowledge, attitude and practices of antibiotic usage among students from Mumbai University. Int J Res Med Sci 6(6):1908. https:// doi.org/10.18203/2320-6012.ijrms20182270 19. Lv B, Zhou Z, Xu G et al (2014) Knowledge, attitudes and practices concerning self-medication with antibiotics among university students in western China. Trop Med Int Health 19:769–779. https://doi.org/10.1111/tmi.12322 20. Mc Nulty CAM, Boyle P, Nichols Clappison P, Davey P (2007) Don't wear me out-The public's knowledge of and attitudes to antibiotic use. J Antimicrob Chemother 59(4):727–738. https:// doi.org/10.1093/jac/dkl558 21. Minen MT, Duquaine D, Marx MA, Weiss D (2010) A survey of knowledge, attitudes, and beliefs of medical students regarding antimicrobial use and resistance. Microb Drug Resist 16(4):285–289. https://doi.org/10.1089/mdr.2010.0009 22. Pan H, Cui B, Zhang D, Farrar J, Law F et al (2012) Prior knowledge, older age, and higher allowance are risk factors for selfmedication with antibiotics among university students in southern China. PLoS ONE 7:e41314. https://doi.org/10.1371/journal.pone.0041314 23. Sanya TE, Titilayo OF, Adisa R, Segun JS (2013) Use of antibiotics among non-medical students in a Nigerian University. Afri Health Sci 13(4):1149–1155. https://doi.org/10.4314/ahs.v13i4.41 24. Sarahroodi S, Arzi A, Sawalha AF, Ashtarinezhad A (2010) Antibiotic self-medication among south Iranian University students. Int J Pharmacol 6(1):48–52. https://doi.org/10.3923/ijp.2010.48.52 25. Sawalha AF (2008) A descriptive study of self-medication practicesamong Palestinian medical and nonmedical university students. Res Social Adm Pharm 4:164–172. https://doi.org/10.1016/j.sapharm.2007.04.004 26. Scaioli G, Gualano MR, Gili R, Masucci S, Bert F, Siliquini R (2015) Antibiotic use: a cross-sectional survey assessing the knowledge, attitudes and practices amongst students of a school of medicine in Italy. PLoS ONE 10:e0122476. https://doi.org/10.1371/journal.pone.0122476 27. Suaifan GA, Shehadeh M, Darwish DA, Al-Ije H, Yousef AM, Darwish RM (2012) A cross-sectional study on knowledge, attitude and behavior related to antibiotic use and resistance among medical and non-medical university students in Jordan. Afr J Pharm Pharmacol 6(10):763–770. https://doi.org/10.5897/AJPP12.080 28. Tomas A, Kusturica MP, Tomic Z, Horvat O, Koprivica DD, Bukumiric D, Sabo A (2017) Self-medication with antibiotics in Serbian households: a case for action? Int J Clin Pharm 39(3):507–513. https://doi.org/10.1007/s11096-017-0461-3 29. World Health Organization (2015) Antibiotic resistance: multicountry public awareness survey. WHO Available: https://apps.who.int/iris/handle/10665/194460 30. World Health Organization (2018a) Central Asian and Eastern European surveillance of antimicrobial resistance. Annual report WHO Available: http://www.euro.who.int/__data/assets/pdf_file/0007/386161/52238-WHO-CAESAR-AR2018_low_V11_web.pdf?ua=1 31. World Health Organization (2018b) WHO report on surveillance of antibiotic consumption: 2016–2018 Early Implementation. Available: https://www.who.int/medicines/areas/rational_use/who-amr-amc-report-20181109.pdf

A REVIEW OF ANTIMICROBIAL RESISTANCE IN POULTRY PRODUCTION - GLOBAL IMPACTS AND ALTERNATIVES

Zoran Ružić[1*], Zdenko Kanački[1], Zorana Kovačević[1], Slobodan Stojanović[1], Dragoljub Marić[1], Srđan Todorović[1], Slobodan Knežević[2], Suzana Vidaković Knežević[2], Dušan Lazić[2]

[1]University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia

[2]Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia

[*]e-mail: ruzicvet@gmail.com

Abstract

Antibiotics are used to fight bacterial infections. Hence, selective pressure gave rise to bacteria resistant to antibiotics. This leaves scientists worried about the danger to human and animal health. Some strategies can be borrowed to reduce the use of antibiotics in chicken farms. Many research has been carried out to look for natural agents with similar beneficial effects such growth promoters. The aim of these alternatives is to maintain a low mortality rate, and a good level of animal yield while preserving the environment and consumer health. Among these, the most popular are probiotics, prebiotics, enzymes, organic acids, immunostimulants, bacteriocins, bacteriophages, phytogenic feed additives, phytoncides, nanoparticles and essential oils (EOs).

Key words: antibiotics, antimicrobial resistance, organic breeding, poultry

Sažetak

Antibiotici se koriste za borbu protiv bakterijskih infekcija. Međutim, selektivni pritisak je doveo do bakterija otpornih na antibiotike. Ovo ostavlja naučnike zabrinutim zbog opasnosti po zdravlje ljudi i životinja. Neke strategije se mogu primeniti kako bi se smanjila upotreba antibiotika na farmama pilića. Sprovedeno je mnogo istraživanja u potrazi za prirodnim agensima sa sličnim blagotvornim efektima promotera rasta. Cilj ovih alternativa je održavanje niske stope mortaliteta, dobrog nivoa prinosa životinja uz očuvanje životne sredine i zdravlja potrošača. Među njima su najpopularniji probiotici, prebiotici, enzimi, organske kiseline, imunostimulansi, bakteriocini, bakteriofagi, fitogeni dodaci hrani, fitoncidi, nanočestice i etarska ulja.

Ključne reči: antibiotic, antimikrobna rezistencija, organska proizvodnja, živinarstvo

Introduction

The discovery of antibiotics was a success in controlling infectious pathologies and increasing feed efficiencies in poultry (Engberg et al., 2000). Antibiotics, either of natural or synthetic origin are used to both prevent proliferation and destroy bacteria. Antibiotics are produced by lower fungi or certain bacteria. They are routinely used to treat and prevent infections in humans and animals. However, scientific evidence suggests that the massive use of these compounds has led to the increasing problem of antibiotic resistance (Forgetta et al., 2012; Furtula et al., 2010; Diarra et al., 2007), and the presence of antibiotics residues in feed and environment (Gonzalez Ronquillo and Angeles Hernandez, 2017; Carvalho and Santos, 2016), compromises human and animal health (Mehdi et al., 2018; Diarra et al., 2010).

Over the past 50 years, the use of antibiotics combined with strict biosecurity and hygiene measures has helped the poultry industry to grow by preventing the negative impacts of many avian diseases (Bermudez, 2003). Antibiotics are not effective against fungal and viral pathogens. They only treat infectious diseases whose causative agents are bacteria. In general, antibiotics are used in phytosanitary treatments, fish farming, animal feed, and human or veterinary medicine where they can be used as a preventive or curative treatment. Antibiotics are classified according to their chemical family, mode of action and the species of bacteria on which they act. Bactericidal antibiotics kill bacteria and bacteriostatics weaken them by inhibiting their proliferation and facilitating their phagocytosis by the immune system. Thus, mortality rate decreases because animals become more resistant (Mehdi et al., 2018).

The first use of antibiotic drugs in poultry can be traced back to 1946 (Moore et al., 1946) and first resistance was reported in food animals by Starr and Reynolds (1951), with concerns about the development of resistance dating back to 1969 (Dibner and Richards, 2005). After the first cases of antibiotic resistant bacterial diseases in humans, recommendations were made for banning the use of antibiotics as growth promoters if drugs are also prescribed for use in human medicine (e.g., penicillins, tetracyclines, and sulfonamides); (Swann et al., 1969).

According to the World Health Organization (WHO), antimicrobial resistance (AMR) is defined as “an increase in the minimum inhibitory concentration of a compound for a previously sensitive strain” (WHO, 2013). Four general mechanisms cause antibiotic resistance: target alteration, drug inactivation, decreased permeability, and increased efflux (Munita and Arias, 2016). It is still uncertain if resistance genes are a result of adaptation through chromosomal mutation (or gene shuffling), or through horizontal gene transfer (or the movement of genetic materials between different organisms), instead of vertical transmission of DNA from parent to offspring

(Yang et al., 2019; Nesme and Simonet, 2015).

There is growing interest in sustainable food production and research is currently being conducted to identify antibiotic alternatives that could support healthy growth and provide defence against pathogenic microbes (Gadde et al., 2017; Sneeringer et al., 2015). Therefore, the broiler industry is now a new leader in management systems that seeks to eliminate the use of antibiotics for the entire broiler lifecycle. The use of antibiotics as growth factors is not allowed in the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) participating countries (ESVAC, 2017).

Reservoirs and Transmission of AMR Bacteria and Genes From Farm-to-Field

AMR genes have been recovered from 30,000 years old permafrost samples, which suggests AMR is an ancient phenomenon, existing before antibiotic usage (D'Costa et al., 2011). Laboratory work also demonstrates that antibiotic resistant strains are very stable even in the absence of antibiotic selection pressure (Gibreel et al., 2005). Consequently, AMR development by pathogenic bacteria and/or commensal (or "friendly") bacteria is a complex interaction and an evolutionary phenomenon (Yang et al., 2019).

The global consumption of antibiotics in human and animal production is estimated between $1x10^5$ and $2x10^5$ tons (Manzetti and Ghisi, 2014). Releasing thereby large quantities of antibiotics into the environment entertains the cycle of biotransformation and bioaccumulation of antibiotics in the environment. According to Manzetti and Ghisi (2014), the most vulnerable ecosystems to antibiotic contamination are confined aquatic ecosystems such as ponds and lakes and soils close to urban sites. Aquatic compartments, such as water and sediments, thus could play an important role in the transfer, evolution and ecology of antibiotic resistance genes (Marti et al., 2014). Large amounts of antibiotics administered to animals are excreted into the environment via urine and feces (Carvalho and Santos, 2016). After metabolic changes in animals, 30% to up 90% of the dose consumed is found in the urine and feces as parent compounds and/or metabolite compounds (Carvalho and Santos, 2016). This makes sewage disposal systems one of the most important routes by which antibiotics can enter in the environment (Gonzalez Ronquillo and Angeles Hernandez, 2017) and contaminate even coastal waters (Chen et al., 2015). Residues of antibiotics present in the water contribute strongly to the maintenance, emergence and dissemination of bacterial populations with a low level of resistance and are ready to evolve towards resistance (Mehdi et al., 2018; Corvaglia, 2006).

In the soil, antibiotics' behaviour differs according to their physicochemical

properties, soil characteristics, as well as climate conditions. Acid rain accelerates the accumulation of antibiotics in animal manure and soil surface while long-lasting rains foster antibiotics' migration in deeper parts of the soil (Pan and Chu, 2017). According to Pan and Chu (2017), antibiotics leaching is higher in sandy soils than in clay and silty soils. Norfloxacin and tetracycline tend to persist on the soil surface while sulfamethazine and erythromycin pose a higher risk for deeper soil layers and groundwater. The soil can be also contaminated by antibiotics in a litter. Animal bedding contains residues of antimicrobial compounds. Residues of bacitracin, salinomycin, penicillin and virginiamycin were detected in chicken litter at concentrations ranging from 0.07 to 66 mg/L (Furtula et al., 2010). When this bedding material is used as nitrogen amendment, the resistant bacteria can live in the soil for several months (Merchant et al., 2012). According to De Liguoro et al. (2003), biotransformation and biodegradation of antibiotics on agricultural sites can take up to 150 days. In addition, antibiotic by-products in the environment remain bioactive and can be potentially more toxic, stable and mobile than their parent compounds (Mehdi et al., 2018; Carvalho and Santos, 2016).

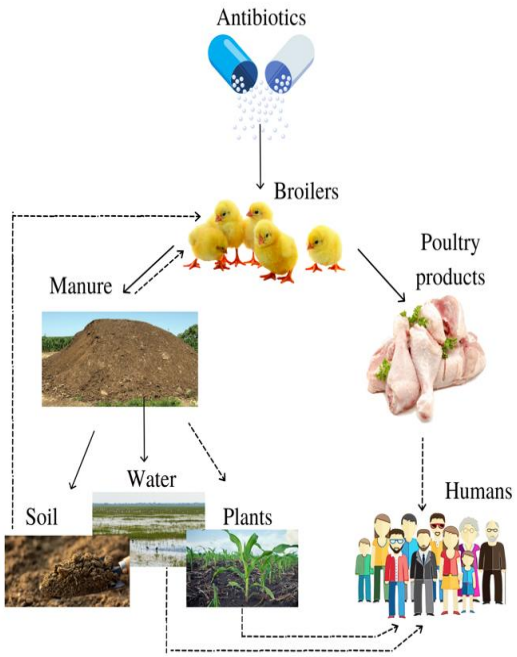


Figure 1. Potential AMR transmission route from broiler chicken antibiotic induction – to flock – to either poultry litter or meat products and the soil-water environment. Solid lines suggest direct transmission, while dotted lines indicate indirect or possible transmission route (According to Yang et al., 2019)

The role of the environment as a transmission route for bacterial pathogens has long been recognized, often associated with fecal contamination of water or organic fertilizer applications (Bengtsson-Palme et al., 2018). Depending on antibiotic properties, significant (e.g., up to 90%) amounts of veterinary antibiotics pass un-degraded through the animal gut to manure (Berendsen et al., 2015; Sarmah et al., 2006). Bacterial pathogens can be introduced to a flock via many routes, including feed, water, air, insects and other pests (Mouttotou et al., 2017; Trampel et al., 2014). Once introduced into a flock, pathogenic bacteria are excreted in the manure, and can survive in the litter (Chen and Jiang, 2014). Therefore, antibiotics, resistance genes, and microorganisms can be transferred from manure to soil (Cook et al., 2014; He et al., 2014). Following land application of poultry litter, antibiotics migrate from soil through runoff, leaching, and particle adsorbed runoff (Leal et al., 2013; Sun et al., 2013; Kay et al., 2004), potentially ending up in soil, surface water, and groundwater (He et al., 2014; Figure 1). Measuring antibiotics in a complex matrix, such as soil, is subject to technical limitations, and studies measuring veterinary pharmaceuticals in soil and water are reviewed elsewhere (Yang et al., 2019; Aga et al., 2016; Dinh et al., 2011; Thiele-Bruhn, 2003).

Numerous routes have been suggested for the introduction of AMR pathogens into the chicken flock, such as horizontal gene transfer from an environmental source to the chicken flock (Krauland et al., 2009), or vertical transmission from breeder to progeny chicks (Pearson et al., 1996). Feed and water can also serve as potential reservoirs and transmit AMR pathogens from the environment to the chicken flock (Perez-Boto et al., 2010; Byrd et al.,1998). Although it is thought that cross contamination of meat products can occur during the slaughter process (Berrang et al., 2001; Berndtson et al., 1996), there is limited information related to the transmission route from one part of contaminated meat to the whole retail product (Yang et al., 2019).

Mechanisms of AMR Gene Transfer

Scientific evidence suggests that the use of antimicrobials in livestock production can promote bacterial resistance in treated animals (O'Brien, 2002). Antibiotic resistance is defined as the ability of microorganisms to proliferate in presence of an antibiotic that generally inhibits or kills microorganisms of the same species. Typically, it takes antibiotic development at least 10 years before certification for general public (Renwick et al., 2016; Ahmad et al., 2011). In contrast, bacteria can evolve resistance within a few hours (Burckhardt and Zimmermann, 2011), making the evolutionary arms race a one-sided competition (Mehdi et al., 2018).

Acquired bacterial resistance is caused by four general mechanisms including inactivation, target alteration, decreased permeability, and increased efflux (Dunning Hotopp, 2011). First, target site changes typically occur from spontaneous mutation of a bacterial gene with selection pressure of antibiotics (Dibner and Richards, 2005). Two examples consist of mutations in RNA polymerase and DNA gyrase which facilitate resistance in rifamycins and quinolones, respectively (Lambert, 2005). Second, target alteration uses a strategy to make the antibiotic ineffective through enzymatic degradation, commonly occurring among aminoglycosides, chloramphenicol, and beta-lactams (Peterson and Kaur, 2018). Third, Gram-negative bacteria can decrease permeability to selectively filter antibiotics from entering the cell membrane (Maria-Neto et al., 2018). Fourth, efflux pumps function mainly to release toxic substances from the bacterium and many of these pumps can transport an extensive variety of compounds (Hedman et al., 2020; Blair et al., 2014; Villagra et al., 2012).

Two fundamental biological pathways that facilitate the evolution and dissemination of resistance include vertical gene transfer (VGT) and horizontal gene transfer (HGT) (Figure 2). First, resistance can occur among a pre-existing phenotypic-resistant bacteria population. Genetic mutations within bacterial genome that promotes AMR can be transferred from parent to daughter cells, via VGT, such as the resistance to fluoroquinolones and oxazolidinones (Munita and Arias, 2016; Caniça et al., 2015; Silva and Guimarães, 2013) (Figure 2A). In the second pathway, genetic mechanisms facilitating resistance can be exchanged between bacterial species, which is also often described as horizontal gene transfer (HGT) (Husnik and McCutcheon, 2018) (Figure 2B). HGT usually manifests through the following three mechanisms: (1) transformation, defined as the exogenous DNA from environment through cell membrane, (2) transduction, defined as gene transfer from one bacterium to another through a viral medium, and (3) conjugation, defined as gene transfer from a donor to a recipient cell through direct cell-to-cell contact mediated by plasmids (Husnik and McCutcheon, 2018, Catry et al., 2003). Transformation and transduction usually occur between microorganisms that are closely phylogenetically related. Whereas, conjugation can occur between different Phyla allowing a promiscuous bacterial transfer of AMR. Plasmids are the most important medium of antibiotic resistant gene (ARG) dispersion. These circular DNA structures (plasmids) are often scaffolds of ARGs and mobile genetic elements (MGEs) (e.g., transposons, integrons, and insertion sequences), facilitating the emergence of multidrug-resistant (MDR) bacteria (Hedman et al., 2020; Moser et al., 2017; von Wintersdor et al., 2016; Zhu et al., 2013; Dunning Hotopp, 2011).

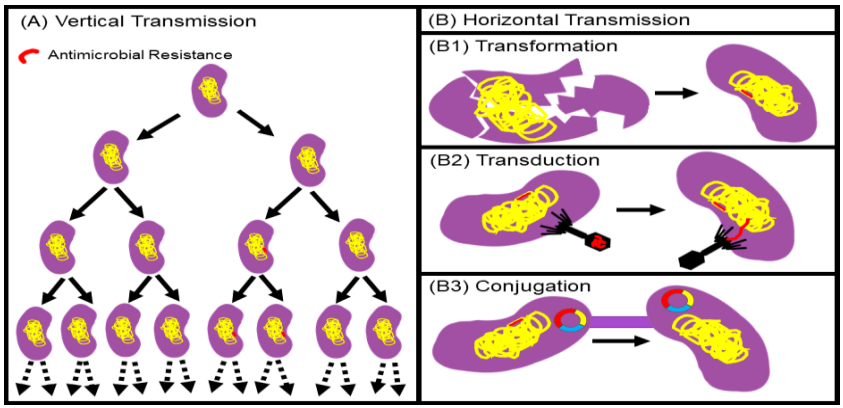


Figure 2. Primary pathways involved in the exchange of genetic information conferring antibiotic resistance consisting of (A) vertical transmission and horizontal transmission (B). (Hedman et al., 2020)

Alternatives to antimicrobials

Consumers' pressure and worries about the harmful effects of antibiotic use and the ban of antibiotics in the EU have prompted researchers to think about alternatives to antibiotics (Diarra and Malouin, 2014). These alternatives aim to maintain a low mortality rate, and a good level of animal yield while preserving the environment and consumer health. Much research has been carried out to look for natural agents with similar beneficial effects to growth promoters. There are indeed several non-therapeutic alternatives that can substitute antibiotics use. Among these, the most popular are probiotics, prebiotics, enzymes, organic acids, immunostimulants, bacteriocins, bacteriophages, phytogenic feed additives, phytocides, nanoparticles and essential oils (EOs) (Hedman et al., 2020).

Prebiotics, probiotics and symbiotics (prebiotic and probiotic combination; Figure 3) are alternative feed additives used to control pathogens in the gut and increase bird performance (Peralta-Sanchez et al., 2019; Murate et al., 2015). Prebiotics are oligosaccharides indigestible by the host animal but are utilized by specific populations of gut microorganisms. Prebiotics enable already present microorganisms to increase their numbers, reduce pathogenic bacteria, increase digestibility, increase minerals and vitamins absorbability, maintain optimal intestinal pH, and maximize nutrients utilization (Mazanko et al., 2018; Kulshreshtha et al., 2014; Figure 3; Wenk, 2000). Prebiotics can affect host health in several ways, such as the production of metabolites like lactic acid, modification of microbial metabolism, and increased cell integrity of the epithelium (Yaqoob et al., 2021; Neupane et al., 2019). Unlike prebiotics, probiotics are microorganisms that can alter

the host health by colonizing the host GIT and providing a more balanced microbiota (Murate et al., 2015; Wenk, 2000). Probiotics can be yeast, bacteria, or fungi based and, unlike antibiotics, do not have the potential to leave a residue. Lactic acid bacteria, which produce antifungal metabolites, are common probiotic bacteria (Londero et al., 2014). Probiotics, also known as direct-fed microbial (DFMs), are classified by FAO/WHO (2001) as "live microorganisms which when administered in adequate amounts confer a health benefit on the host." Probiotics specific to broilers include the genera *Lactobacillu*s, *Streptococcus*, *Bacillus*, *Bifidobacterium*, *Enterococcus*, *Aspergillus*, *Candida*, and *Saccharomyces* (Kabir, 2009). The beneficial effects of probiotics include improved performance, modulating the intestinal microbiota, inhibiting pathogens, improved intestinal integrity, immunomodulation, and improving microbiological and sensory characteristics of broiler meat (Abd El-Hack et al., 2022; Alagawany et al., 2021; El-Saadony et al., 2021).

A broad category of feed additives commonly researched in poultry is phytogenic (PFAs), intended to improve gut health and function (Alcicek et al., 2004). PFAs are plant-derived compounds such as EOs, spices, safe natural compounds, and herbs intended to provide a health benefit when added to feed (Ashour et al., 2021). Typical examples are rosemary derivatives, oregano, thyme, sage, cinnamon, citrus, pepper, and anise (Mountzouris, 2016). The efficacy of phytogenics can depend on numerous factors, including composition, inclusion level in the feed, bird genetics, and feed composition (Ferdous et al., 2019). PFAs may promote a healthy gut and improve performance through various mechanisms: antioxidative and antimicrobial properties, improved palatability, improved digestion, growth promotion, and improved gut health (Alcicek et al., 2004). Studies on palatability are inconclusive, but the PFAs may improve feed quality due to antioxidative properties and slow bacterial and fungal growth (Abd El-Hack et al., 2022; Burt, 2004; Soliman and Badeaa, 2002; Lambert et al., 2001).

Essential oil is (EOs) classified as the plant essence or benzene or terpene derivatives obtained through water and/or steam distillation (Abd El-Hack et al., 2022; El-Tarabily et al., 2021). EOs are valuable due to their antimicrobial, antiviral, antioxidant and antiparasitic capabilities. Many plant sources of EOs have been studied for feed additive efficacy. EOs improve poultry production by increasing the activity of digestive enzymes, lowering the number of fermentation products, lowering the number of pathogens, improving nutrient digestion, enhancing intestinal accessibility of important nutrients, and enhancing antioxidant capacity and immune function (Abd El-Hack et al., 2022; Alagawany et al., 2021; Brenes and Roura, 2010).

Organic acids are organic substances that have an acidic pH. Carboxylic acids such as lactic acid, propionic acid, acetic acid, formic acid, sorbic acid, citric acid, oxalic acid, uric acid, and butyric acid are the most prominent types (Dibner and Buttin, 2002). Organic acids are not antibiotics, but when used in conjunction with excellent nutrition, management, and biosecurity procedures, they can help poultry maintain intestinal health, improving livability, feed conversion ratios, weight gain, live weight, and immunological responses (Abd El-Hack et al., 2022; Adil et al., 2011).

The addition of exogenous enzymes has become the standard for improving digestibility and efficiency of nutrient utilization (Ravindran, 2013). According to Pariza and Cook (2010), to digest food, all animals need enzymes, which are either created by the animal or by bacteria in the digestive tract. The digestive system, on the other hand, isn't perfect. Therefore, supplementing the animal feed with appropriate enzymes improves digestive efficiency (Munir and Maqsood, 2013). Enzyme supplementation helps to reduce the amount of nutrient excretion, which, if ignored, can result in extra costs to the farmer, feed supplier, and the environment (Ali and Abdelaziz, 2018; Sheppy, 2010). Exogenous enzymes are essential to the reduction of feed costs.

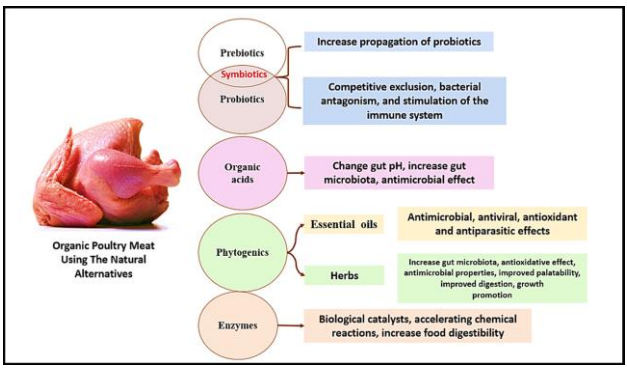


Figure 3. Types of the natural alternatives for antibiotics and their mode of action (Abd El-Hack et al., 2022)

Concluding remarks

The majority of antimicrobial use is for food animal production (Van Boeckel et al., 2019). Poultry encompasses the most abundant and fastest-growing per capita livestock and is one of the most common sources of multi-resistant bacteria (Elwinger et al., 2016; Dibner et al., 2005). Over the years, antibiotics have played an important

role in fighting infectious diseases and stimulating poultry growth. Scientific evidence suggests that their large-scale use has led to antibiotic resistance and residues in the food and environment, particularly the aquatic ecosystem, which can lead to public health problems. Many trials of potential alternatives to antibiotics have shown very relevant results. These alternatives give equal or better effects to antibiotics (namely good livestock performance), reduce mortality rates and protect the environment and consumer health. The application of the results generated by these studies in feed industries, as well as livestock breeders and veterinary practice is very appealing. Some studies show that antibiotic use can be dropped or reduced. It should also be noted that some trials have shown the efficacy of vaccination of broiler chickens as a prophylactic treatment against necrotic enteritis induced by *C. perfringens* (Caly et al., 2015). Another way of research in the future is to test the interactive effect of using combinations of these alternatives. The aim will be to maintain a high level of viability and optimum productivity in poultry on antibiotic-free farms.

References

1. Abd El-Hack M. E., El-Saadony M. T., Salem H. M., El-Tahan A. M., Soliman M. M., Youssef G. et al. 2022. Alternatives to antibiotics for organic poultry production: types, modes of action and impacts on bird's health and production. Poultry Science, 101(4):101696.2. Adil S., Banday M. T., Bhat G. A., Mir M. S. 2011. Alternative strategies to antibiotic growth promoters−a review. Journal of Veterinary Medicine, 6:76. 3. Aga D. S., Lenczewski M., Snow D., Muurinen J., Sallach J. B., Wallace J. S. 2016. Challenges in the measurement of antibiotics and in evaluating their impacts in agroecosystems: a critical review. Journal of Environmental Quality, 45:407–19. 4. Ahmad V., Khan M. S., Jamal Q. M. S., Alzohairy M. A., Al Karaawi M. A., Siddiqui M. U. 2017. Antimicrobial potential of bacteriocins: In therapy, agriculture and food preservation. International Journal of Antimicrobial Agents, 49:1–11. 5. Alagawany M., Madkour M., El-Saadony M. T., Reda F. M. 2021. Paenibacillus polymyxa (LM31) as a new feed additive: antioxidant and antimicrobial activity and its effects on growth, blood biochemistry, and intestinal bacterial populations of growing Japanese quail. Animal Feed Science and Technology, 276:114920. 6. Alcicek A. H. M. E. T., Bozkurt M., Cabuk M. 2004. The effect of a mixture of herbal essential oils, an organic acid or a probiotic on broiler performance. South African Journal of Animal Science, 34:217–22. 7. Ali N., Abdelaziz M. 2018. Effect of feed restriction with supplementation of probiotic with enzymes preparation on performance, carcass characteristics and economic traits of broiler chickens during finisher period. Egyptian Journal of Nutrition and Feeds, 21:243–54. 8. Ashour E. A., Farsi R. M., Alaidaroos B. A., Abdel-Moneim A. M. E., El-Saadony M. T., Osman A. O., Abou Sayed-Ahmed E. T., Albaqami N. M., Shafi M. E., Taha A. E., Abd El-Hack M. E. 2021. Impacts of dietary supplementation of pyocyanin powder on growth performance, carcase traits, blood chemistry, meat quality and gut microbial activity of broilers. Italian Journal of Animal Science, 20:1357–72. 9. Bengtsson-Palme J., Kristiansson E., Larsson D.G.J. 2018. Environmental factors influencing the development and spread of antibiotic resistance. FEMS Microbiology Reviews, 42:fux053. 10. Berendsen B.J.A., Wegh R.S., Memelink J., Zuidema T., Stolker L.A.M. 2015. The analysis of animal faeces as a tool to monitor antibiotic usage. Talanta, 132:258–68. 11. Bermudez A.J. 2003. Principles of disease prevention: diagnosis and control. In: Saif YM, editor. Diseases of poultry. Ames, Ia, USA: Iowas State University Press; p. 360. 12. Berndtson E., Danielsson-Tham M. L., Engvall A. 1996. Campylobacter incidence on a chicken farm and the spread of Campylobacter during the slaughter process. International Journal of Food Microbiology, 32:35–47. 13. Berrang M. E., Buhr R. J., Cason J. A., Dickens, J. A. 2001. Broiler carcass contamination with Campylobacter from feces during defeathering. Journal of Food Protection, 64:2063–6. 14. Blair J. M., Richmond G. E., Piddock L. J. 2014. Multidrug efflux pumps in Gram-negative bacteria and their role in antibiotic resistance. Future Microbiology, 9:1165–77. 15. Brenes A., Roura E. 2010. Essential oils in poultry nutrition: main effects and modes of action. Animal Feed Science and Technology, 158:1–14. 16. Burckhardt I., Zimmermann S. 2011. Using Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry To Detect Carbapenem Resistance within 1 to 2.5 Hours. Journal of Clinical Microbiology, 49:3321–4. 17. Burt S. 2004. Essential oils: their antibacterial properties and potential applications in foods − a review. International Journal of Food Microbiology, 94:223–53. 18. Byrd J. A., Corrier D. E., Hume M. E., Bailey R. H. Stanker L. H., Hargis B. M. 1998. Effect of feed withdrawal on Campylobacter in the crops of market-age broiler chickens. Avian Disease, 42:802–6. 19. Caly D. L., D'Inca R., Auclair E., Drider D. 2015. Alternatives to antibiotics to prevent necrotic enteritis in broiler chickens: a microbiologist's perspective. Frontiers in Microbiology, 6:1336. 20. Caniça M., Manageiro V., Jones-Dias D., Clemente L., Gomes-Neves E., Poeta P., Dias E., Ferreira E. 2015. Current perspectives on the dynamics of antibiotic resistance in different reservoirs. Research in Microbiology, 166:594–600. 21. Carvalho I.T., Santos L. 2016. Antibiotics in the aquatic environments: a review of the European scenario. Environment International, 94:736e57. 22. Catry B., Laevens H., Devriese L. A., Opsomer G., Kruif A. 2003. Antimicrobial resistance in livestock. Journal of Veterinary Pharmacology and Therapeutics, 26:81–93. 23. Chen H., Liu S., Xu X. R., Zhou G.J., Liu S.S., Yue W.Z., et al. 2015. Antibiotics in the coastal environment of the hailing bay region, south China sea: spatial distribution, source analysis and ecological risks. Marine Pollution

Bulletin, 95:365e73. 24. Chen Z., Jiang X. 2014. Microbiological safety of chicken litter or chicken litter-based organic fertilizers: a review. Agriculture, 4:1–29. 25. Cook K.L., Netthisinghe A.M.P., Gilfillen R.A. 2014. Detection of pathogens, indicators, and antibiotic resistance genes after land application of poultry litter. Journal of Environmental Quality, 43:1546–58. 26. Corvaglia A.R. 2006. Role des residus D'antibiotiques dans L'environnement hydrique sur La selection et La diffusion De bacteries resistantes des genres "aeromonas", "acinetobacter" et "Legionella". These De Doctorat: Univsity Geneve no Sc. 3796. 27. D'Costa V. M., King C. E., Kalan L., Morar M., Sung W. W. L., Schwarz C., et al. 2011. Antibiotic resistance is ancient. Nature, 477:457–61. 28. De Liguoro M, Cibin V, Capolongo F, Halling-Sorensen B, Montesissa C. Use of oxytetracycline and tylosin in intensive calf farming: evaluation of transfer to manure and soil. Chemosphere 2003;52:203e12. 29. Diarra M. S., Malouin F. 2014. Antibiotics in canadian poultry productions and anticipated alternatives. Frontiers in Microbiology,5:282. 30. Diarra M.S., Rempel H., Champagne J., Masson L., Pritchard J., Topp E. 2010. Distribution of antimicrobial resistance and virulence genes in enterococcus spp. and characterization of isolates from broiler chickens. Applied and Environmental Microbiology, 76:8033e43. 31. Diarra M.S., Silversides F.G., Diarrassouba F., Pritchard J., Masson L., Brousseau R., et al. 2007. Impact of feed supplementation with antimicrobial agents on growth performance of broiler chickens, clostridium perfringens and enterococcus counts, and antibiotic resistance phenotypes and distribution of antimicrobial resistance determinants in Escherichia Coli isolates. Applied and Environmental Microbiology, 73:6566e76. 32. Dibner J. J., Richards J. D. 2005. Antibiotic growth promoters in agriculture: history and mode of action. Poultry Science, 84:634–43. 33. Dibner, J. J., and P. Buttin. 2002. Use of organic acid as a model to study the impact of gut microflora on nutrition and metabolism. Journal of Applied Poultry Research, 11:453–463. 34. Dinh Q. T., Alliot F., Moreau-Guigon E., Eurin J., Chevreuil M., Labadie P. 2011. Measurement of trace levels of antibiotics in river water using online enrichment and triple-quadrupole LC–MS/MS. Talanta, 85:1238–45. 35. Dunning Hotopp J. C. 2011. Horizontal gene transfer between bacteria and animals. Trends in Genetics, 27:157–63. 36. El-Saadony M. T., Zabermawi N. M., Burollus M. A., Shafi M. E., Alagawany M., Yehia NAskar., A. M., Alsafy S. A., Noreldin A. E.,. Khafaga A. F, Dhama K., Elnesr S. S., Elwan H. A. M., Di Cerbo A., El-Tarabily K. A., Abd El-Hack M. E. 2021. Nutritional aspects and health benefits of bioactive plant compounds against infectious diseases: a review. Food Reviews International, 37:1–23. 37. El-Tarabily K. A., El-Saadony M. T., Alagawany M., Arif M.,. Batiha G. E, Khafaga A. F., Elwan H. A., Elnesr S. S., Abd El-Hack M. E. 2021. Using essential oils to overcome bacterial biofilm formation and their antimicrobial resistance. Saudi Journal of Biological Sciences, 28:5145–56. 38. Elwinger K., Fisher C., Jeroch H., Sauveur B., Tiller H., Whitehead C. C. 2016. A brief history of poultry nutrition over the last hundred years. World's Poultry Science Journal, 72:701–20. 39. Engberg R.M., Hedemann M.S., Leser T.D., Jensen B.B. 2000. Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. Poultry Science, 79:1311e9. 40. ESVAC. Sales of veterinary antimicrobial agents in 30 european countries in 2015.Trends from 2010 to 2015. Seventh Esvac Report. Ema/184855/2017. 2017. 41. Ferdous M. F., Arefin M. S., Rahman M. M.,. Ripon M. M. R, Rashid M. H.,. Sultana M. R, Rafiq K. 2019. Beneficial effects of probiotic and phytobiotic as growth promoter alternative to antibiotic for safe broiler production. Journal of Advanced Veterinary and Animal Research. 6:409–15. 42. Forgetta V., Rempel H., Malouin F., Vaillancourt Jr R., Topp E., Dewar K., et al. 2012. Pathogenic and multidrug-resistant Escherichia fergusonii from broiler chicken. Poultry Science, 91:512e25. 43. Furtula V., Farrell E.G., Diarrassouba F., Rempel H., Pritchard J., Diarra M.S., et al. 2010. Veterinary pharmaceuticals and antibiotic resistance of Escherichia Coli isolates in poultry litter from commercial farms and controlled feeding trials. Poultry Science, 89:180e8. 44. Gadde U., Kim W., Oh S., Lillehoj H. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: a review. Animal Health Research Reviews, 18:26–45. 45. Gibreel A., Kos V. N., Keelan M., Trieber C. A., Levesque S., Michaud S. et al. 2005. Macrolide resistance in Campylobacter jejuni and Campylobacter coli: molecular mechanism and stability of the resistance phenotype. Antimicrobial Agents and Chemotherapy, 49:2753–9. 46. Gonzalez Ronquillo M., Angeles Hernandez J.C. 2017. Antibiotic and synthetic growth promoters in animal diets: review of impact and analytical methods. Food Control, 72:255e67. Part B. 47. He L.Y., Liu Y.S., Su H.C., Zhao J.L., Liu S.S., Chen J. et al. 2014. Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. Environmental Science & Technology, 48:13120–13129. 48. Hedman H. D., Vasco K. A., Zhang L. 2020. A Review of Antimicrobial Resistance in Poultry Farming within Low-Resource Settings. Animals, 10:1264. 49. Husnik F., McCutcheon J. P. 2018. Functional horizontal gene transfer from bacteria to eukaryotes. Nature Reviews Microbiology, 16:67–79. 50. Kabir S. M. L. 2009. The role of probiotics in the poultry industry. International Journal of Molecular Sciences, 10:3531–46. 51. Kay P., Blackwell P.A., Boxall A. 2004. Fate of veterinary antibiotics in a macroporous tile drained clay soil. Environmental Toxicology and Chemistry, 23:1136–44. 52. Krauland M. G., Marsh J. W., Paterson D. L., Harrison L. H. 2009. Integron-mediated multidrug resistance in a global collection of nontyphoidal Salmonella enterica isolates. Emerging Infectious Diseases, 15:388–96. 53. Kulshreshtha G., Rathgeber B., Stratton G., Thomas N., Evans F., Critchley A., Prithiviraj B. 2014. Feed supplementation with red seaweeds, Chondrus crispus and Sarcodiotheca gaudichaudii, affects performance, egg quality, and gut microbiota of layer hens. Poultry Science, 93:2991–3001. 54. Lambert P. 2005. Bacterial resistance to antibiotics: Modified target sites. Advanced Drug Delivery Reviews, 57:1471–85. 55. Lambert R. J. W., Skandamis P. N, Coote P. J., Nychas G-JE. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. Journal of Applied Microbiology, 91:453-462. 56. Leal R.M.P., Alleoni L.R.F., Tornisielo V.L., Regitano J.B. 2013. Sorption of fluoroquinolones and sulfonamides in 13 Brazilian soils. Chemosphere, 92:979–85. 57. Londero A., Leon Pelaez M. A., Diosma G., De Antoni G. L., Abraham A. G., Garrote G. L. 2014. Fermented whey as poultryfeed additive to prevent fungal contamination. Journal of the Science of Food and Agriculture, 94:3189–94. 58. Manzetti S., Ghisi R. 2014. The environmental release and fate of antibiotics. Marine Pollution Bulletin, 79:7e15. 59. Maria-Neto S., de Almeida K. C., Macedo M. L. R., Franco O. L. 2015. Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside. Biochimica et Biophysica Acta, 1848:3078–88. 60. Marti E., Variatza E., Balcazar J.L. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends in Microbiology, 22:36e41. 61. Mazanko M. S., Gorlov I. F., Prazdnova E. V., Makarenko M. S., Usatov A. V., Bren A. B., Chistyakov V. A., Tutelyan A. V., Komarova Z. B., Mosolova N. I., Pilipenko D. N.. 2018. Bacillus probiotic supplementations improve laying performance, egg quality, hatching of laying hens, and sperm quality of roosters. Probiotics and Antimicrobial Proteins, 10:367–73. 62. Mehdi Y., Létourneau-Montminy M. P., Gaucher M. L., Chorfi Y., Suresh G., Rouissi T., Kaur Brar S., Côté C., Avalos Ramirez A., Godbout S. 2018. Use of antibiotics in broiler production: Global impacts and alternatives. Animal Nutrition, 4(2):170-8. 63. Merchant L.E., Rempel H., Forge T., Kannangara T., Bittman S., Delaquis P., et al. 2012. Characterization of antibiotic-resistant and potentially pathogenic Escherichia Coli from soil fertilized with litter of broiler chickens fed antimicrobial supplemented diets. Canadian Journal of Microbiology, 58:1084e98. 64. Moore P. R., Evenson A., Luckey T. D., McCoy E., Elvehjem C. A., Hart E. B.

1946. Use of sulfasuxidine, streptothricin, and streptomycin in nutritional studies with the chick. Journal of Biological Chemistry, 165:437–41. 65. Moser K. A., Zhang L., Spicknall I., Braykov N. P., Levey K., Marrs C. F., Foxman B., Trueba G., Cevallos W., Goldstick J., et al. 2017. The role of mobile genetic elements in the spread of antimicrobial resistant escherichiacoli from chickens to humans in small-scale production poultry operations in rural ecuador. American Journal of Epidemiology, 95:1–10. 66. Mountzouris K. C. 2016. Phytogenic and probiotic feed additives for broilers: evidence for growth performance links with gut performance indices. Pages 107-116 in Proceedings of the 2016 World Nutrition Forum. Erber Ag. 67. Mouttotou N., Ahmad S., Kamran Z., Koutoulis K.C. 2017. "Prevalence, risks and antibiotic resistance of Salmonella in poultry production chain," in Current Topics in Salmonella and Salmonellosis. Chapter 12, ed. Mares, M (Rijeka:InTech) 215–234. 68. Munir K., Maqsood S. 2013. A review on role of exogenous enzyme supplementation in poultry production. Emirates Journal of Food and Agriculture, 25:66–80. 69. Munita J. M., Arias C. A. 2016. Mechanisms of antibiotic resistance. Microbiology Spectrum, 4(2): VMBF-0016-2015. 70. Murate L. S, F. G., Paiao A. M., de Almeida A., Berchieri Jr, Shimokomaki M. 2015. Efficacy of prebiotics, probiotics, and synbiotics on laying hens and broilers challenged with Salmonella enteritidis. The Journal of Poultry Science, 52:52–6. 71. Nesme J., Simonet P. 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environmental Microbiology, 17:913–30. 72. Neupane D., Nepali D. B, Devkota N., Sharma M. P., Kadaria I. P. 2019. Effect of probiotics on production and egg quality of dual-purpose chicken at Kathmundu in Nepal. Bangladesh Journal of Animal Science, 48:29–35. 73. O'Brien TF. 2002. Emergence, spread, and environmental effect of antimicrobial resistance: how use of an antimicrobial anywhere can increase resistance to any antimicrobial anywhere else. Clinical Infectious Diseases, 34(3):S78e84. 74. Pan M., Chu L.M. 2017. Leaching behavior of veterinary antibiotics in animal manureapplied soils. Science of The Total Environment, 579:466e73. 75. Pariza M. W., Cook M. 2010. Determining the safety of enzymes used in animal feed. Regulatory Toxicology and Pharmacology, 56:332–42. 76. Pearson A. D., Greenwood M. H., Feltham R. K. Healing T. D., Donalsdon J., Jones D. M. et al. 1996. Microbial ecology of Campylobacter jejuni in a United Kingdom chicken supply chain: intermittent common source, vertical transmission, and amplification by flock propagation. Applied and Environmental Microbiology, 62:4614–20. 77. Peralta-Sanchez J. M., Martín-Platero A. M., Ariza-Romero J. J., Rabelo-Ruiz M., Zurita-Gonalez M. J., Banos A., Martínez-Bueno M. 2019. Egg production in poultry farming is improved by probiotic bacteria. Frontiers in Microbiology, 10:1042. 78. Perez-Boto D., Garcia-Pena F. J., Abad-Moreno J. C., Hurtado-Pizarro M. D., Perez-Cobo, I., Echeita, M. A. (2010). Drinking water as the source of Campylobacter coli infection in grandparent heavy breeders. Avian Pathology, 39:483–7. 79. Peterson E., Kaur P. 2018. Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens. Frontiers in Microbiology, 9:1-21. 80. Ravindran V. 2013. Feed enzymes: the science, practice, and metabolic realities. Journal of Applied Poultry Research, 22:628–36. 81. Renwick M. J., Brogan D. M., Mossialos E. 2016. A systematic review and critical assessment of incentive strategies for discovery and development of novel antibiotics. The Journal of Antibiotics, 69:73–88. 82. Sarmah A.K., Meyer, M.T., Boxall A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinaryantibiotics (VAs) in the environment. Chemosphere, 65:725–59. 83. Sheppy C. 2010. Enzymes in Farm Animal Nutrition, CABI Publishing, Oxfordshire, UK, 1–10. 84. Silva N. C. C., Guimarães F. F., Manzi M.P., Budri P.E., Gómez-Sanz E., Benito D., Langoni H., Rall V. L. M., Torres C. 2013. Molecular characterization and clonal diversity of methicillin-susceptible Staphylococcus aureus in milk of cows with mastitis in Brazil. Journal of Dairy Science, 96:6856–62. 85. Sneeringer S., MacDonald J., Key N., McBride W., Mathews K. 2015. Economics of Antibiotic Use in U.S. Livestock Production, ERR-200, U.S. Washington,DC: United States Department of Agriculture, Economic Research Service. 86. Soliman K. M., Badeaa R. I. 2002. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. Food and Chemical Toxicology, 40:1669–75. 87. Starr M. P., Reynolds, D. M. 1951. Streptomycin resistance of coliform bacteria from turkeys fed streptomycin. In Proceedings of the 51st General Meeting, Society of American Bacteriology, Chicago, IL. 88. Sun P., Barmaz D., Cabrera M.L., Pavlostathis S.G., Huang C.H. 2013. Detection and quantification of ionophore antibiotics in runoff, soil and poultry litter. Journal of Chromatography A, 1312:10–17. 89. Swann M. M., Baxter K. L., Field H. I. 1969. Report of the joint committee on the use of antibiotics in animal husbandry and veterinary medicine. MHSO; London. 90. Thiele-Bruhn S. 2003. Pharmaceutical antibiotic compounds in soils–a review. Journal of Plant Nutrition and Soil Science, 166:145–67. 91. Trampel D.W., Holder T.G., Gast R.K. 2014. Integrated farm management to prevent Salmonella enteritidis contamination of eggs, Journal of Applied Poultry Research, 23:353–65. 92. Van Boeckel T. P., Pires J., Silvester R., Zhao C., Song J., Criscuolo N. G., Gilbert M., Bonhoe er S., Laxminarayan R. 2019. Global trends in antimicrobial resistance in animals in low- and middle-income countries. Science, 365;eaaw1944. 93. Villagra N. A., Fuentes J. A., Jofre M. R., Hidalgo A. A., Garcia P., Mora G. C. 2012. The carbon source influences the efflux pump-mediated antimicrobial resistance in clinically important Gram-negative bacteria. Journal of Antimicrobial Chemotherapy, 67:921–7. 94. von Wintersdor C. J. H., Penders J., van Niekerk J. M., Mills N. D., Majumder S., van Alphen L. B., Savelkoul P. H. M., Wols P. F. G. 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Frontiers in Microbiology, 7. 95. Wenk C. 2000. Recent advances in animal feed additives such as metabolic modifiers, antimicrobial agents, probiotics, enzymes and highly available minerals. Asian-Australasian Journal of Animal Sciences, 13:86–95. 96. World Health Organization [WHO] (2013). Antimicrobial Resistance (factsheet no 194). Geneva: World Health Organization 97. Yang Y., Ashworth A. J., Willett C., Cook K., Upadhyay A., Owens P. R., Ricke S. C., DeBruyn J., M., Moore Jr. P. A. 2019. Review of Antibiotic Resistance, Ecology, Dissemination, and Mitigation in U.S. Broiler Poultry Systems. Frontiers in Microbiology, 10:2639. 98. Yaqoob M., Abd El-Hack M. E., Hassan F., El-Saadony M. T., Khafaga A., Batiha G., Yehia N., Elnesr S., Alagawany M., El-Tarabily K. A., Wang M. 2021. The potential mechanistic insights and future implications for the effect of prebiotics on poultry performance, gut microbiome, and intestinal morphology. Poultry Science, 100:101143. 99. Zhu Y. G., Johnson T. A., Su, J. Q., Qiao M., Guo G. X., Stedtfeld R. D., Hashsham S. A., Tiedje J. M. 2013. Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proceedings of the National Academy of Sciences of the United States of America, 110:3435–40.

RESISTANCE MECHANISMS OF *PSEUDOMONAS AERUGINOSA* TO FLUOROQUINOLONES

Selma Pintarić[1*], Zrinka Štritof[1], Marija Cvetnić[1], Lana Hadžić[2], Branka Šeol Martinec[1]

[1]*University of Zagreb, Faculty of Veterinary Medicine, Department of Microbiology and Infectious Diseases, with Clinic, Zagreb, Croatia*

[2]*Laboratory for food, water and animal feed microbiology, PI 'Veterinary Institute', Bihać, Bosnia and Herzegovina*

[*]*e-mail: selma.pintaric@vef.unizg.hr*

Abstract

The bacterium *Pseudomonas aeruginosa* is the cause of difficult-to-treat opportunistic infections in humans and animals with innate resistance to a variety of antimicrobial drugs commonly used in human and veterinary medicine. This innate resistance significantly limits the range of antimicrobials effective against this bacterium to carboxypenicillins (carbenicillin, ticarcillin), ureidopenicillins (piperacillin), third (cefoperazone, ceftazidime) and fourth generation cephalosporins (cefepime, cefpirome), carbapenems, aminoglycosides, fluoroquinolones, and polymyxins. All those antimicrobial groups are categorized by the World Health Organization as critically important antimicrobials in human medicine, so their use in veterinary medicine is strongly discouraged.

In addition, *P. aeruginosa* is rapidly evolving new resistance mechanisms by acquiring resistance genes or adapting to environmental conditions, which is manifested by overexpression of efflux pumps, decreased expression of porin for antimicrobial entry, and modified target sites for antimicrobials. Of concern is the fact that multiple resistance mechanisms can manifest simultaneously in a single isolate, resulting in a multidrug-resistant phenotype.

Fluoroquinolones are synthetic antimicrobial drugs with broad spectrum of antibacterial activity against Gram-negative pathogens, including *P. aeruginosa*. Their efficacy depends on the applied dose, i.e., the concentration reached in the tissues. In addition to better antibacterial efficacy, the optimal dose and treatment duration prevents the selection of resistant bacterial strains and the spread of resistance.

In this paper, we provide an overview of the resistance mechanisms of *P. aeruginosa* to fluoroquinolones: decreased permeability, increased activity of efflux pumps, target site modifications, and acquisition of plasmids carrying resistance genes.

Key words: acquired resistance, fluoroquinolones, innate resistance, *Pseudomonas aeruginosa,* resistance mechanisms

Introduction

Infections with multidrug-resistant bacteria are an increasing problem in both human and veterinary medicine. *Pseudomonas aeruginosa* (*P. aeruginosa*), an opportunistic, ubiquitous bacterial pathogen, is of particular concern due to its antimicrobial resistance and ability to cause various infections in animals and humans, including otitis externa, dermatitis, conjunctivitis, lower urinary tract infections, septicemia, and bacterial endocarditis (Markey et al., 2013; Hirsh et al., 2004). In human medicine, *P. aeruginosa* is one of the most common causes of nosocomial infections (Labovská, 2021; Mesaros et al., 2007).

Unfortunately, the treatment of *P. aeruginosa* infections has become a major challenge due to its ability to resist many of the currently available antimicrobials. In addition, the overuse of antimicrobials can lead to the development and selection of multidrug-resistant *P. aeruginosa* strains, causing the failure of empirical antimicrobial therapy against this bacterium (Pang et al., 2019). The World Health Organization (WHO) has listed carbapenem-resistant *P. aeruginosa* as one of the bacterial species for which new antimicrobials are urgently needed (World Health Organization, 2017).

Generally, the main mechanisms of resistance in *P. aeruginosa* can be classified into intrinsic, acquired, and adaptive resistance (Pang et al., 2019). Intrinsic resistance of *P. aeruginosa* involves low outer membrane permeability, production and derepression of chromosomal AmpC β-lactamase, and the presence of numerous genes encoding various multidrug resistance efflux pumps (Poole, 2001; Hancock, 1998; Livermore, 1995).

Apart from its high intrinsic resistance, *P. aeruginosa* has a great capacity to acquire other resistance mechanisms, which can be achieved either by horizontal transfer of resistance genes or by mutational changes and include acquisition of resistance genes, target site alterations, increased efflux pump activity, decreased porin expression, and production of antibiotic-hydrolyzing enzymes. These mechanisms can be present simultaneously in one strain, resulting in a multidrug-resistant phenotype (Strateva and Yordanov, 2009).

Adaptive resistance in *P. aeruginosa* is induced by environmental stimuli and involves the formation of a biofilm that serves as a diffusion barrier to limit antimicrobial access to bacterial cells (Pang et al., 2019).

Mechanisms of fluoroquinolone action

Fluoroquinolones include a large group of synthetic antimicrobial drugs that play a significant role in the treatment of bacterial infections with a wide range of antibacterial activity against Gram-negative pathogens. The first representative of quinolones is nalidixic acid, which has been used since 1962. The addition of a fluorine molecule at position 6 increased antibacterial activity, and the entire group was named ”fluoroquinolones”. The addition of piperazinyl or pyrrolidinyl group at position 7 improved the effect of fluoroquinolones on *P. aeruginosa* (Martinez et al., 2006). The first fluoroquinolone approved for use in veterinary medicine was enrofloxacin (Giguère et al., 2013).

Fluoroquinolones are an important class of antimicrobials for the treatment of *P. aeruginosa* infections, with ciprofloxacin and levofloxacin being the most commonly used in human medicine (Zhao et al., 2020). Fluoroquinolones are bactericidal antimicrobial drugs whose efficacy depends on the dose applied, i.e., the concentration reached in tissues. In addition to better antibacterial activity, the optimal dose also prevents the selection of resistant strains and the spread of resistance (Giguère et al., 2013).

Fluoroquinolones belong to the group of antimicrobial drugs that inhibit bacterial nucleic acid synthesis by interfering with the enzyme topoisomerase II (DNA gyrase) and topoisomerase IV (Giguère et al., 2013; Martinez et al., 2006). DNA gyrase is a tetramer consisting of two GyrA and two GyrB subunits encoded by the *gyr*A and *gyr*B genes. Topoisomerase IV has a similar structure and consists of two ParC and two ParE subunits encoded by the *par*C and *par*E genes (Giguère et al., 2013). The main target of fluoroquinolones in gram-negative bacteria is DNA gyrase, especially its subunit GyrA (Van Bambeke et al., 2005).

The main disadvantage of fluoroquinolones is that their use often leads to in the development and selection of resistant strains (Giguère et al., 2013). This can be prevented by appropriate dosing that leads to adequate concentration of the drug in the tissues. Namely, the higher the concentration of the drug, the higher the percentage of susceptible microorganisms that the drug can eliminate. The dose of the drug is based on the minimum inhibitory concentration (MIC) value for a particular microbe. When the bacterium is exposed to a lower concentration of fluoroquinolones than the effective one, fewer bacterial cells are susceptible, so after treatment all cells with a higher MIC will survive. Such bacteria have the potential to become the dominant population (Dudley et al., 1991). Therefore, it is extremely important to adjust the dose of fluoroquinolones to a minimum inhibitory concentration that is different for each microbe, and this is especially true for *P. aeruginosa* (Giguère et al., 2013;

Prescott et al., 2000). According to Cole et al. (2008), for bacteria with a minimum inhibitory concentration of 0.51-0.64 μg/ml, enrofloxacin should be dosed at 20 mg/kg (high end of the enrofloxacin dose range). On this basis, according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2015), all intermediate susceptible isolates should be considered resistant because adequate tissue levels cannot be achieved even with a maximum enrofloxacin dose.

Intrinsic fluoroquinolone resistance

Intrinsic resistance can be defined as a trait that is universally shared within a bacterial species, is independent of previous antimicrobial exposure, and is not related to horizontal gene transfer (Reygaert, 2018). *Pseudomonas aeruginosa* has a high degree of intrinsic resistance due to low outer membrane permeability, efflux systems that pump antimicrobials out of the cell, and production of antibiotic inactivating enzymes that confer resistance to a very wide range of antimicrobial drugs: most penicillins, first- and second-generation cephalosporins, some third-generation cephalosporins (cefotaxime, ceftriaxone), macrolides, chloramphenicol, some aminoglycosides (streptomycin, neomycin, kanamycin and spectinomycin), tetracyclines, trimethoprim and sulfonamides, quinolones, and some fluoroquinolones (Livermore et al., 2001). Therefore, the choice of antimicrobial drugs for the treatment of pseudomonas infections is limited to ureidopenicillins (piperacillin), carboxypenicillins (carbenicillin, ticarcillin), third-generation (cefoperazone, ceftazidime) and fourth-generation (cefepime, cefpirome) cephalosporins, carbapenems, aminoglycosides, fluoroquinolones, and polymyxins (Giguère et al., 2013). All of these antimicrobial groups are classified by WHO as critically important antimicrobials in human medicine, and their use in veterinary medicine is therefore strongly discouraged (World Health Organization, 2019).

Outer membrane permeability

Most antimicrobials used to treat *P. aeruginosa* infections must be able to penetrate the cell membrane to reach their sites of action. Quinolone antimicrobials interfere with DNA replication by inhibiting DNA gyrase and topoisomerase IV (Giguère et al., 2013). They are small molecules that pass through the cell wall through water channels surrounded by porin proteins. In *P. aeruginosa*, this non-specific permeability of the wall is very low, so antimicrobials pass through the porins extremely slowly and with difficulty. For example, pseudomonas porins pass 10-100 times fewer molecules than *E. coli* porins (Yoshimura and Nikaido, 1982).

In general, porins can be divided into four classes: the nonspecific porins for the

slow diffusion of small hydrophilic molecules; specific porins with specific sites for the binding of specific molecules; gated porins, which are ion-regulated outer membrane proteins responsible for the uptake of ion complexes; and efflux porins, which are components of efflux pumps (Pang et al., 2019).

Porin OprF is the predominant porin in all strains of *P. aeruginosa*, whose function is nonspecific and serves as the main entry point for various molecules into the bacterial cell, but it has low efficiency for antimicrobial penetration (Nikaido, 1994). Porin OprF can fold into two conformers: the closed conformer with two domains and the open channel conformer with one domain. The closed conformer is the predominant structure of OprF porin, which may explain why the permeability of the outer membrane of *P. aeruginosa* is much lower than that of other bacteria (Sugawara et al., 2006).

In addition to OprF, pseudomonas possess several porins with specific functions, of which the OprD porin, which contains the binding sites for carbapenems, is most important for antimicrobial uptake. The absence of this porin in *P. aeruginosa* leads to resistance to imipenem (Hancock, 1998).

Efflux pumps

Decreased accumulation of drugs in bacterial cells due to active efflux is an important mechanism of antimicrobial resistance in gram-negative bacteria. The term "efflux" refers to the active elimination of a toxic substance from a cell via transmembrane systems consisting mainly of three proteins. The first protein is a transporter located in the cell membrane and functions as an energy-dependent pump. The second protein, located on the cell wall, is a porin, and the third protein, located in the periplasmic space, connects the first two by linking them to form a channel through which substances are expelled from the periplasmic space and cytoplasm into the extracellular space (Strateva and Yordanov, 2009).

Many different substances, such as antiseptics, dyes, detergents, and organic solvents, as well as antimicrobials, are ejected via pumps (Lister et al., 2009).

Genome analysis of the *P. aeruginosa* reference strain PAO1 revealed the existence of 12 efflux systems (Stover et al., 2000), four of which are important for antimicrobial resistance: MexA-MexB-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY. Each of them has the ability to bind and release different molecules and thus different antimicrobial drugs.

The MexC-MexD-OprJ and MexE-MexF-OprN pumps extrude members of different groups of antimicrobial drugs and thus may be involved in the development of a multidrug-resistant phenotype. These pumps become active through mutation of

regulatory genes and play no role in innate resistance (Hancock and Brinkman, 2002).

The MexA-MexB-OprM and MexX-MexY pumps are involved in both innate and acquired resistance to various antimicrobials (Morita et al., 2001; Masuda et al., 2000a; Masuda et al., 2000b). Both pumps eject antimicrobials from the cell through the same porin. Indeed, the MexX-MexY pump does not have a porin gene in its operon but binds to the OprM porin encoded by a gene in the MexA-MexB-OprM pump operon (Masuda et al., 2000a; Mine et al., 1999). This gene is spontaneously expressed, and therefore the porin OprM is constantly active in the bacterial cell, regardless of the presence of antimicrobials and other substrates of these pumps. Therefore, this porin is responsible for the innate resistance of pseudomonas to many antimicrobials, especially to older generations of fluoroquinolones (LI et al., 1994), which was confirmed by a study in which removal of the OprM gene showed 10 to 1000 times greater susceptibility of *P. aeruginosa* to antimicrobial drugs (Hancock and Brinkman, 2002).

Fluoroquinolones are a good substrate for all of the above pumps, but in terms of innate resistance, they are only affected by the MexA-MexB-OprM efflux pump (Masuda et al., 2000b). However, Tejedor et al. (2003) reported that the MexA-MexB-OprM pump does not act equally on all fluoroquinolones. The more lipophilic fluoroquinolones such as difloxacin are more affected by the overexpression of this efflux pump than the less lipophilic molecules such as ciprofloxacin. Thus, it is evident that basal expression of the MexA-MexB-OprM pump is not sufficient to confer innate resistance to all fluoroquinolones but is responsible for resistance to more lipophilic fluoroquinolone antimicrobials. However, an important feature of efflux pumps is that they protect bacteria from low concentrations of antimicrobials, especially fluoroquinolones, and thus may allow the emergence and selection of resistant strains with higher resistance levels (Hawkey, 2003; Lomovskaya et al., 1999).

In the last few decades, the possibilities of inhibiting the action of efflux pumps have been intensively investigated, which would improve the efficacy of various antimicrobials, especially fluoroquinolones as a substrate for all pumps. Lomovskaya et al. (1999) found that inhibition of pumps would significantly reduce the level of innate resistance, as well as the frequency of emergence of fluoroquinolone-resistant strains. Since efflux pumps are found not only in bacterial cells but also in host cells (humans and animals), an inhibitor that does not interfere with their action is required (Lomovskaya and Watkins, 2001). However, the use of efflux pump inhibitors has emerged as a potential therapeutic strategy for the treatment of *P. aeruginosa* infections (Askoura et al., 2011).

Antibiotic-inactivating enzymes

The production of enzymes that inactivate or modify antimicrobials is one of the most important mechanisms of intrinsic resistance in bacteria. Many antimicrobials have chemical bonds that are susceptible to hydrolysis by enzymes commonly produced by *P. aeruginosa*, such as β-lactamases and aminoglycoside-modifying enzymes (Poole, 2005; Hancock and Speert, 2000). Like many Gram-negative bacteria, *P. aeruginosa* possesses an inducible, naturally occurring *amp*C gene that encodes cephalosporinase, which confers low-level resistance to aminopenicillins and first-generation cephalosporins (Wolter and Lister, 2013; Livermore, 1995). Fluoroquinolones are not affected by this enzyme, but expression of the *amp*C gene may be increased during exposure to some fluoroquinolones, such as ciprofloxacin (Wolter et al., 2007).

Acquired fluoroquinolone resistance

In addition to the high intrinsic resistance of *P. aeruginosa*, acquired resistance also contributes greatly to the selection of multidrug-resistant strains, making eradication of this microorganism difficult (Henrichfreise et al., 2007). There are several acquired mechanisms responsible for *P. aeruginosa* resistance to fluoroquinolones: change in cell wall permeability, increased activity of efflux pumps, change in the target site of action of fluoroquinolones, acquisition of plasmids carrying resistance genes (Giguère et al., 2013). Most commonly, resistance arises through site-specific mutations and overexpression of efflux pumps (Zhao et al., 2020).

Decreased outer membrane permeability

In order to act on the target site, fluoroquinolones must penetrate the bacterial cell, which in gram-negative bacteria means they must pass through the cell wall. To do this, they use porins with non-specific functions, the most common of which is the porin OprF. Alterations in this porin or its deficiency generally have little effect on the occurrence of resistance, but in combination with other mechanisms can cause an increase in MIC (Van Bambeke et al., 2005). The change in susceptibility to fluoroquinolones due to OprF porin deficiency is usually accompanied by decreased susceptibility to other antimicrobial agents that use this porin to enter the cell.

Increased activity of efflux pumps

Because fluoroquinolones are a good substrate for all efflux pumps described in *P. aeruginosa*, increased activity of any pump contributes to the development of

resistance. In addition, resistance to other unrelated antimicrobials (cephalosporins, carbapenems), which are also substrates of pumps, is also characteristic (Giguère et al., 2013).

The change in susceptibility is mainly influenced by the MexA-MexB-OprM pump, whose increased activity leads to resistance to fluoroquinolones and all beta-lactam antimicrobials except imipenem. The genes encoding the proteins of this pump form an operon controlled by the transcriptional regulator MexR, which is encoded by the *mex*R regulatory gene. When MexR is nonfunctional due to mutations in the *mex*R gene, MexAB-OprM overexpression occurs (Scoffone et al., 2021; Masuda et al., 2000a). Mutations as well as the selection of mutants are usually the consequence of therapy with fluoroquinolones, penicillins and cephalosporins (Ziha-Zarifi et al., 1999).

For the innate resistance of pseudomonas, the efflux pump MexC-MexD-OprJ is irrelevant because its expression is stimulated by a mutation in the *nfx*B gene. NfxB is the negative regulator of MexCD-OprJ and inhibits the action of the pump (Poole, 2001). Therefore, the efflux pump MexCD-OprJ is expressed only in *nfx*B mutants. These mutants showed different levels of resistance to the antimicrobials effluxed by MexCD-OprJ, such as chloramphenicol, erythromycin, fluoroquinolones, and tetracyclines (Scoffone et al., 2021; Masuda et al., 1996). Mutations occur most frequently with the use of newer fluoroquinolones such as norfloxacin and ciprofloxacin (Köhler et al., 1997).

The efflux pump MexE-MexF-OprN is expressed only in strains with a mutation in the *nfx*C gene characterized by a multidrug-resistant phenotype. Its substrates are fluoroquinolones, chloramphenicol, and trimethoprim. Imipenem resistance occurs in strains with expressed pumps because mutation of the *nfx*C gene is associated with decreased expression of the porin OprD, which is crucial for imipenem entry into the cell (Poole, 2001).

Mutations in the *mex*Z gene cause increased activity of the MexXY efflux pump, which can lead to resistance to fluoroquinolones, but this pump has a greater and more significant effect on aminoglycosides (Llanes et al., 2004; Vogne et al., 2004).

Modification of the fluoroquinolone target sites

Mutations in chromosomal genes encoding the enzymes DNA gyrase and topoisomerase IV play an important role in the spread of fluoroquinolone resistance. Mutations occur most frequently in a stepwise manner in the quinolone resistance-determining region (QRDR), which alters the amino acid composition of the enzyme

(Hawkey, 2003; Drlica and Zhao, 1997). In gram-negative bacteria, DNA gyrase is the primary target of fluoroquinolone activity, so the first step in the development of resistance is changes in the enzyme subunit of GyrA, which is encoded by the *gyr*A gene (Hooper, 1999). Mutations in the *gyr*B gene, which encodes the DNA gyrase subunit GyrB, can also lead to quinolone resistance, but the frequency of these mutations is much lower than that of mutations in the *gyr*A gene (Vila et al., 1996).

Plasmid-mediated fluoroquinolone resistance

Plasmid-mediated quinolone resistance (PMQR) is considered a common resistance mechanism in gram-negative bacilli. To date, three PMQR-mediated mechanisms of fluoroquinolone resistance have been recognized, which include *qnr* genes (encoding Qnr proteins that are blocking drug targets), acetyltransferase aac(6')-Ib-cr, which is a variant of an enzyme involved in aminoglycoside modification and resistance, and mobile efflux pumps such as QepA and OqxAB (Saki et al., 2022; Rodríguez-Martínez et al., 2016). Only few data are available on the prevalence of PMQR genes among clinical isolates of *P. aeruginosa* worldwide, although a few examples have been reported (Khan et al., 2020; Molapour et al., 2020; Al-Marjani, 2014).

Adaptive fluoroquinolone resistance

Unlike intrinsic and acquired resistance, which are characterized by an irreversible phenotype that is independent of the presence of the antimicrobial agent or the environmental conditions surrounding the microorganism and can be transmitted vertically to the next generations, adaptive resistance has an unstable property and usually reverts when the environmental stimulus is removed (Pang et al., 2019; Fernández et al., 2011). Adaptive resistance can be defined as the induction of resistance to one or more antimicrobials in response to the presence of a specific signal (Fernández et al., 2011). Adaptive resistance increases the ability of a bacterium to survive an antimicrobial attack due to transient changes in gene and/or protein expression in response to an environmental stimulus (Pang et al., 2019).

Due to its transient nature, this type of resistance is difficult to detect. However, adaptive resistance may be responsible for the clinical failure of some antimicrobial regimens, especially when antimicrobial concentrations are in the subinhibitory range during treatment (Fernández et al., 2011).

In *P. aeruginosa*, the best characterized mechanisms of adaptive resistance are the formation of a biofilm and the differentiation of persister cells (Pang et al., 2019; Taylor et al., 2014).

Biofilm-mediated resistance

A biofilm can be described as a microbial sessile community characterized by cells irreversibly attached to a surface or interface or to each other and embedded in a matrix of extracellular polymeric substances that they have produced themselves. By forming a biofilm, bacteria, including *P. aeruginosa*, protect themselves from host defenses, disinfectants, and antimicrobials. Bacteria in biofilm are much more resistant to antimicrobials than planktonic forms (Dincer et al., 2020), but antimicrobial susceptibility can be rapidly restored if bacteria lose biofilm protection (Pang et al., 2019). General mechanisms of biofilm-mediated resistance include prevention of antimicrobial entry, slow growth of biofilm cells due to differences in nutrient and oxygen availability within biofilms, induction of an adaptive stress response that acts as a preventive factor for cellular damage rather than repair factor, quorum sensing as a process of the cell-to-cell interaction that regulates gene expression, and differentiation of persister cells (Pang et al., 2019; Singh et al., 2017).

Persister cells in antimicrobial resistance

Another difficulty in the treating of *P. aeruginosa* infections is the formation of bacterial persister cells, a subpopulation of cells that are not genetically resistant to antimicrobials but can survive exposure to a bactericidal drug concentration (Balaban et al., 2019; Pang et al., 2019). Unlike resistant cells, persister bacterial cells cannot replicate in the presence of the drug any better than the non-persister cells but are killed at a lower rate than susceptible cells (Balaban et al., 2019).

Many stress conditions have been shown to trigger persistence, including nutrient deficiency, high cell number, acid stress, exposure to immune cells, and exposure to antimicrobials. In the case of drug-induced persistence, instead of killing cells, a bactericidal antimicrobial becomes bacteriostatic to a subpopulation of cells that respond to the antimicrobial activity, for example, by activating a stress response that allows them to survive (Balaban et al., 2019). It has been observed that *P. aeruginosa* increases persister cell differentiation in chronic infections that have been associated with frequent and long-term antimicrobial treatment, and that these persister cells are able to remain viable and repopulate biofilms (Van den Bergh et al., 2017; Mulcahy et al., 2010). For example, Dudley et al. (1991) described that ciprofloxacin is 99% bactericidal at MIC concentrations or higher, but at subinhibitory concentrations, even if ciprofloxacin is bactericidal, it does not kill all cells at the first exposure, leading to re-growth of resistant subpopulations that can be observed after 4-6 hours. A second exposure to fluoroquinolones had no or very little bactericidal effect on these strains. Thus, those cells that were not killed by the first exposure are

highly adaptively resistant to subsequent treatment (Fernández et al., 2011; Dudley et al., 1991).

Fluoroquinolone resistance of *P. aeruginosa* in veterinary medicine in Croatia

The first report of resistance to fluoroquinolones in veterinary medicine in Croatia was published in 2002 with isolates of *P. aeruginosa* from dogs. Antimicrobial susceptibility testing showed that among the three fluoroquinolones tested, ciprofloxacin was the most effective with 3.8% of resistant isolates, followed by marbofloxacin with 4.4% of resistant isolates (Šeol et al., 2002). Enrofloxacin showed lower activity with 26.2% of resistant isolates. Resistance rates reported nine years later were higher with 8.7% of ciprofloxacin-resistant and 51.9% of enrofloxacin-resistant isolates (Mekić et al., 2011). The *P. aeruginosa* isolates included in the later study were all from the ears of dogs with otitis externa, and it is known that ear isolates tend to have higher rates of resistance to various antimicrobials, including fluoroquinolones (Harada et al., 2012). Another study was conducted in 2017 comparing two methods (disk diffusion and microdilution) for fluoroquinolone susceptibility testing of *P. aeruginosa* isolates from different samples of dogs (Pintarić et al., 2017). For marbofloxacin and ciprofloxacin, there was no statistically significant disagreement between the results of the two methods. The resistance rates obtained with microdilution were similar to previous studies and were 8.9% for marbofloxacin and 2.2% for ciprofloxacin, respectively. On the other hand, the authors reported statistically significant disagreement between the results obtained with microdilution and disk diffusion testing for enrofloxacin. Most of the disagreements were due to the fact that the disk diffusion test overestimated the number of susceptible strains compared with the microdilution test, leading the authors to conclude that the use of the disk diffusion test to evaluate the enrofloxacin susceptibility of *P. aeruginosa* strains may result in inappropriate and ineffective therapy. The resistance rate determined by microdilution was 15.6%, but the study reported a high percentage of intermediate susceptible isolates (75.6%) (Pintarić et al., 2017).

Concluding remarks

Pseudomonas aeruginosa continues to be extremely important pathogen causing serious infections in animals and humans and posing an increasing challenge for their treatment. Antimicrobial resistance, including fluoroquinolone resistance, in *P. aeruginosa* can occur in intrinsic, acquired, and adaptive form. Mechanisms of intrinsic resistance present in this species prevent the action of some fluoroquinolones

such as norfloxacin and difloxacin, limiting the choice of fluoroquinolones for the treatment of infections, especially in veterinary medicine. In addition, pseudomonas has a remarkable ability to develop or acquire other resistance mechanisms, as well as to adapt to environmental conditions (e.g., exposure to antimicrobials) by forming biofilms and persisting cells that are resistant to antimicrobials. Multiple resistance mechanisms may be present in the same bacterial cell, and infections caused by such multidrug-resistant strains may be virtually impossible to treat.

References

1. Al-Marjani M. F. 2014. Presence of *qnr* gene in environmental and clinical *Pseudomonas aeruginosa* isolates in Baghdad. International Journal of Current Microbiology and Applied Sciences. 3(7): 853-857. 2. Askoura M., Mottawea W., Abujamel T., Taher I. 2011. Efflux pump inhibitors (EPIs) as new antimicrobial agents against *Pseudomonas aeruginosa*. Libyan Journal of Medicine 6:1, 5870. 3. Balaban N. Q., Helaine S., Lewis K., Ackermann M., Aldridge B., Andersson D. I., Brynildsen M. P., Bumann D., Camilli A., Collins J. J., Dehio C., Fortune S., Ghigo J.-M., Hardt W.-D., Harms A., Heinemann M., Hung D. T., Jenal U., Levin B. R., Michiels J., Storz G., Tan M.-W., Tenson T., Van Melderen L., Zinkernagel A. 2019. Definitions and guidelines for research on antibiotic persistence. Nature Reviews Microbiology 17: 441–448. 4. CLSI. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. 2015. 3rd ed. CLSI supplement VET01S. Wayne, PA: Clinical and Laboratory Standards Institute. 5. Cole L. K., Papich M. G., Kwochka K. W., Hillier A., Smeak D. D., Lehman A. M. 2008. Plasma and ear tissue concentrations of enrofloxacin and its metabolite ciprofloxacin in dogs with chronic end-stage otitis externa after intravenous administration of enrofloxacin. Veterinary Dermatology. 20: 51-59. 6. Dincer S., Uslu F. M., Delik A. 2020. Antibiotic resistance in biofilm, in Bacterial Biofilms, Ed. S. Dincer, M. S. Özdenefe, A. Arkut, IntechOpen, London. 7. Drlica K., Zhao X. 1997. DNA Gyrase, Topoisomerase IV, and the 4-Quinolones. Microbiology and Molecular Biology Reviews. 61: 377-392. 8. Dudley M. N., Blaser J., Gilbert D., Mayer K. H., Zinner S. H. 1991. Combination therapy with ciprofloxacin plus azlocillin vs. *Pseudomonas aeruginosa*: effect of simultaneous vs. staggered dosing in an in vitro model of infection. Journal of Infectious Diseases. 164: 499–506. 9. Fernández L., Breidenstein E. B., Hancock R. E. 2011. Creeping baselines and adaptive resistance to antibiotics. Drug Resistance Updates. 14: 1-21. 10. Giguère S., Prescott J. F., Dowling P.M. 2013. Antimicrobial therapy in veterinary medicine. 5th Edition, Wiley Blackwell. 11. Hancock R. E. W. 1998. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. Clinical Infectious Diseases. 27: 93-99. 12. Hancock R. E. W., Brinkman F. S. L. 2002. Function of pseudomonas porins in uptake and efflux. Annual Review of Microbiology. 56: 17-38. 13. Hancock R. E., Speert D. P. 2000. Antibiotic resistance in *Pseudomonas aeruginosa*: mechanisms and impact on treatment. Drug Resistance Updates. 3(4):247-255. 14. Harada K., Arima S., Niina A., Kataoka Y., Takahashi T. 2012. Characterization of *Pseudomonas aeruginosa* isolates from dogs and cats in Japan: current status of antimicrobial resistance and prevailing resistance mechanisms. Microbiology and Immunology. 56: 123-127. 15. Hawkey P. M. 2003. Mechanisms of quinolone action and microbial response. Journal of Antimicrobial Chemotherapy. 51: 29-35. 16. Henrichfreise B., Wiegand I., Pfister W., Wiedemann B. 2007. Resistance mechanisms of multiresistant *Pseudomonas aeruginosa* strains from Germany and correlation with hypermutation. Antimicrobial Agents and Chemotherapy. 51: 4062-4070. 17. Hirsh D. C., Maclachlan N. J., Walker R. L. 2004. Veterinary Microbiology (2nd ed.), Blackwell Publishing, pp. 122-124. 18. Hooper D. C. 1999. Mode of action of fluoroquinolones. Drugs. 58: 6-10. 19. Khan M., Summers S., Rice S., Stapleton F., Willcox M., Subedi D. 2020. Acquired fluoroquinolone resistance genes in corneal isolates of *Pseudomonas aeruginosa*. Infection, genetics and evolution. 85:104574. 20. Köhler T., Michéa-Hamzehpour M., Henze U., Gotoh N., Kocjancic Curty L., Pechere J.-C. 1997. Characterization of MexE-MexF-OprN, a positively regulated multidrug efflux system of *Pseudomonas aeruginosa*. Molecular Microbiology. 23: 345-354. 21. Labovská S. 2021. *Pseudomonas aeruginosa* as a cause of nosocomial Infections. In *Pseudomonas aeruginosa* - Biofilm Formation, Infections and Treatments. Ed. T. Das, IntechOpen, London. 22. Li X.-Z., Livermore D. M., Nikaido H. 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrobial Agents and Chemotherapy. 38: 1732-1741. 23. Lister P. D., Wolter D. J., Hanson N. D. 2009. Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. Clinical Microbiology Reviews. 22: 582-610. 24. Livermore D. M. 1995. β-Lactamases in laboratory and clinical resistance. Clinical Microbiology Reviews. 8(4):557-84. 25. Livermore D. M., Winstanley T. G., Shannon K. P. 2001. Interpretative reading: recognizing the unusual and inferring resistance mechanisms from resistance phenotypes. Journal of Antimicrobial Chemotherapy. 48: 87-102. 26. Llanes C., Hocquet D., Vogne C., Benali-Baitich D., Neuwirth C., Plésiat P. 2004. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB-OprM and MexXY efflux pumps simultaneously. Antimicrobial Agents and Chemotherapy. 48: 1797-1802. 27. Lomovskaya O., Lee A., Hoshino K., Ishida H., Mistry A., Warren M. S., Boyer E., Chamberland S., Lee V. J. 1999. Use of a genetic approach to evaluate the consequences of inhibition of efflux pumps in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 43: 1340-1346. 28. Lomovskaya O., Watkins W. 2001. Inhibition of efflux pumps as a novel approach to combat drug resistance in bacteria. Journal of Molecular Microbiology and Biotechnology. 3: 225-236. 29. Markey B., Leonard F., Archambault M., Cullinane A., Maguire D. 2013. Clinical Veterinary Microbiology. 2nd ed. Mosby Elsevier, Edinburgh, London, New York, Oxford, Philadelphia, St Louis, Sydney, Toronto, pp. 275-284. 30. Martinez M., McDermott P., Walker R. 2006. Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. The Veterinary Journal. 172: 10-28. 31. Masuda N., Gotoh N., Ohya S., Nishino T. 1996. Quantitative correlation between susceptibility and OprJ production in NfxB mutants of *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 40(4): 909-13. 32. Masuda N., Sakagawa E., Ohya S., Gotoh N., Tsujimoto H., Nishino T. 2000a. Contribution of the

MexX-MexY-OprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 44: 2242-2246. 33. Masuda N., Sakagawa E., Ohya S., Gotoh N., Tsujimoto H., Nishino T. 2000b. Substrate specificities of MexAB-OprM, MexCD-OprJ and MexXY-OprM efflux pumps in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 44: 3322-3327. 34. Mekić S., Matanović K., Šeol B. 2011. Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates from dogs with otitis externa. Veterinary Record. 169: 125. 35. Mesaros N., Nordmann P., Plesiat P., Roussel-Delvallez M., Van Eldere J., Glupczynski Y., Van Laethem Y., Jacobs F., Lebecque P., Malfroot A., Tulkens P. M., Van Bambeke F. 2007. *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. Clinical Microbiology and Infection. 13: 560-578. 36. Mine T., Morita Y., Kataoka A., Mizushima T., Tsuchiya T. 1999. Expression in *Escherichia coli* of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 43: 415-417. 37. Molapour A., Peymani A., Saffarain P., Habibollah-Pourzereshki N., Rashvand P. 2020. Plasmid-mediated quinolone resistance in *Pseudomonas aeruginosa* isolated from burn patients in Tehran, Iran. Infectious Disorders - Drug Targets. 20:49-55. 38. Morita Y., Kimura N., Mima T., Mizushima T., Tsuchiya T. 2001. Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1. The Journal of General and Applied Microbiology. 47: 27-32. 39. Mulcahy L. R., Burns J. L., Lory S., Lewis K. 2010. Emergence of *Pseudomonas aeruginosa* strains producing high levels of persister cells in patients with cystic fibrosis. Journal of Bacteriology 192: 6191–9. 40. Nikaido H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science. 264: 382-388. 41. 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. 42. Pintarić S., Matanović K., Šeol Martinec B. 2017. Fluoroquinolone susceptibility in *Pseudomonas aeruginosa* isolates from dogs - comparing disk diffusion and microdilution methods. Veterinarski Arhiv. 87: 291-300. 43. Poole K. 2001. Multidrug efflux pumps and antimicrobial resistance in *Pseudomonas aeruginosa* and related organisms. Journal of Molecular Microbiology and Biotechnology. 3: 255-264. 44. Poole K. 2005. Aminoglycoside resistance in *Pseudomonas aeruginosa*. Antimicrobial Agents and Chemotherapy. 49: 479-487. 45. Prescott J. F., Baggot J. D., Walker R. D. 2000. Antimicrobial therapy in veterinary medicine. 3rd Edition. Iowa State University Press. 46. Reygaert W. C. 2018. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS microbiology. 4(3): 482–501. 47. Rodríguez-Martínez J. M., Machuca J., Eliecer Cano M., Calvo J., Martínez-Martínez L., Pascual A. 2016. Plasmid-mediated quinolone resistance: Two decades on. Drug resistance updates. 29:13-29. 48. Saki M., Sheikh A. F., Seyed-Mohammadi S., Dezfuli A. A. Z., Shahin M., Tabasi M., Veisi H., Keshavarzi R., Khani P. 2022. Occurrence of plasmid-mediated quinolone resistance genes in *Pseudomonas aeruginosa* strains isolated from clinical specimens in southwest Iran: a multicentral study. Scientific Reports. 12: 2296. 48. Scoffone V. C., Trespidi G., Barbieri G., Irudal S., Perrin E., Buroni S. 2021. Role of RND efflux pumps in drug resistance of cystic fibrosis pathogens. Antibiotics. 10: 863. 50. Singh S., Singh S. K., Chowdhury I., Singh R. 2017. Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. The Open Microbiology Journal. 11: 53-62. 51. Stover C. K., Pham X. Q., Erwin A. L., Mizoguchi S. D., Warrener P., Hickey M. J., Brinkman F. S., Hufnagle W. O., Kowalik D. J., Lagrou M., Garber R. L., Goltry L., Tolentino E., Westbrock-Wadman S., Yuan Y., Brody L. L., Coulter S. N., Folger K. R., Kas A., Larbig K., Lim R., Smith K., Spencer D., Wong G. K., Wu Z., Paulsen I. T., Reizer J., Saier M. H., Hancock R. E., Lory S., Olson M. V. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature. 406: 959-964 52. Strateva T., Yordanov D. 2009. *Pseudomonas aeruginosa* – a phenomenon of bacterial resistance. Journal of Medical Microbiology. 58: 1133-1148. 52. Sugawara E., Nestorovich E. M., Bezrukov S. M., Nikaido H. 2006. *Pseudomonas aeruginosa* porin OprF exists in two different conformations. Journal of Biological Chemistry. 281: 16220–16229. 54. Šeol B., Naglić T., Madić J., Bedeković M. 2002. In vitro antimicrobial susceptibility of 183 *Pseudomonas aeruginosa* strains isolated from dogs to selected antipseudomonal agents. Journal of Veterinary Medicine. 49: 188-192. 55. Wolter D. J., Lister P. D. 2013. Mechanisms of beta-lactam resistance among *Pseudomonas aeruginosa*. Current Pharmaceutical Design. 19: 209–222. 56. Tejedor M. T., Martin J. L., Navia M., Freixes J., Vila J. 2003. Mechanisms of fluoroquinolone resistance in *Pseudomonas aeruginosa* isolates from canine infections. Veterinary Microbiology. 94: 295-301. 57. Van Bambeke F., Michot J.-M., Van Eldere J., Tulkens P. M. 2005. Quinolones in 2005: an update. Clinical Microbiology and Infection. 11: 256-280. 58. Van den Bergh B., Fauvart M., Michiels J. 2017. Formation, physiology, ecology, evolution and clinical importance of bacterial persisters. FEMS Microbiology Reviews 41: 219–251. 59. Vila J., Ruiz J., Goñi P., Jimenez De Anta M. T. 1996. Detection of mutations in *par*C in quinolone-resistant clinical isolates of *Escherichia coli*. Antimicrobial Agents and Chemotherapy. 40: 491-493. 60. Vogne C., Ramos Aires J., Bailly C., Hocquet D., Plésiat P. 2004. Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Antimicrobial Agents and Chemotherapy. 48: 1676-1680. 61. Wolter D. J., Lister P. D. 2013. Mechanisms of beta-lactam resistance among *Pseudomonas aeruginosa*. Current Pharmaceutical Design. 19: 209–222. 62. Wolter D. J., Schmidtke A. J., Hanson N. D., Lister P. D. 2007. Increased expression of *amp*C in *Pseudomonas aeruginosa* mutants selected with ciprofloxacin. Antimicrobial Agents and Chemotherapy. 63. World Health Organization. 2017. WHO publishes list of bacteria for which new antibiotics are urgently needed. Available at: https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed. Accessed 10.6.2022. 64. Yoshimura F., Nikaido H. 1982. Permeability of *Pseudomonas aeruginosa* outer membrane to hydrophilic solutes. Journal of Bacteriology. 152: 636-642. 65. Zhao L., Wang S., Li X., He X., Jian L. 2020. Development of in vitro resistance to fluoroquinolones in *Pseudomonas aeruginosa*. Antimicrobial Resistance and Infection Control. 9: 124. 66. Ziha-Zarifi I., Llanes C., Köhler T., Pechere J.-C., Plésiat P. 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrobial Agents and Chemotherapy. 43: 287-291.

MONITORING OF ANTIMICROBIAL RESISTANCE UNDER DIRECTIVE 2020/1729 IN EU COUNTRIES

Dalibor Todorović[1*], Marko Pajić[1], Đorđe Marjanović[2], Dragana Ljubojević Pelić[1], Suzana Vidaković Knežević[1], Biljana Đurđević[1], Maja Velhner[1]

[1]*Scientific Veterinary Institute "Novi Sad", Novi Sad, Serbia*

[2]*University of Belgrade, Faculty of Veterinary Medicine, Department of Pharmacology, Belgrade, Serbia*

[*]*e-mail: dalibor@niv.ns.ac.rs*

Abstract

Monitoring of antimicrobial resistance is mandatory in all EU member states and is carried out according to the Decision of the European Commission No. 2020/1729. Member states are required to test annually the resistance of 170 isolates of *Escehrichia coli*, *Salmonella* spp., *Campylobacter jejuni*/*coli* and *Enterococcus faecalis*/*fecium*, by determining the minimum inhibitory concentration of antibiotic that inhibits bacterial growth (MIC). Also, they are obliged to test 300 isolates of *Escherichia coli* and *Salmonella* spp. for the production of ESBL, AmpC, or CP enzymes. Monitoring shall be carried out in accordance with the procedures described by the European Committee for Antimicrobial Susceptibility Testing (EUCAST), according to the international reference method ISO 20776-1: 2021 (E) entitled "Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices - Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases". Directive EU2020/1729 stipulates that isolates should come from healthy animals, so for these reasons, sampling at the slaughter line in slaughterhouses, retail outlets, as well as on disease-free farms is most appropriate.

Key words: antimicrobial resistance, monitoring, food, microdilution, EU directives

Introduction

According to the European food safety authority (EFSA), the number of people with infections caused by multidrug-resistant bacteria is increasing every year. Public health is most concerned about increasing the resistance of bacteria to antibiotics used in human medicine, such as fluoroquinolones, third and fourth generation cephalosporins, carbapenems and colistin. For these reasons, it is very

important to monitor antibiotic resistance in indicator bacteria in the food production chain, which also includes testing the mechanisms of resistance, which can see the risks of spreading "super" bacteria in humans and animals (EFSA Journal, 2021).

Monitoring and reporting on the occurrence of resistance in bacteria that pose a threat to public health began in 2003 in the countries of the European Union (EU) according to Directive 2003/99/EC. This directive stipulated that EU member states must assess and analyze the movement and sources of antimicrobial resistance in their territory and submit an annual report to the EU Commission, which includes all data collected in accordance with that directive (Directive 2003/99/EC). The EU Commission Decision of 2013 introduced Directive 2013/652/EU, which lays down detailed rules for harmonized monitoring of antimicrobial resistance and reporting in zoonotic and commensal bacteria originating from domestic animals, and from food originating from animals used for human consumption (Directive 2013/652/EU).

In 2019, the European Food Safety Authority (EFSA) published a report "Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from foodproducing animals and food" recommending certain changes to the current monitoring of antimicrobial resistance under the Directive 2013/652/EU. These changes concerned the animal populations for food production and the categories of food to be sampled, the sampling plan to be followed, the types of zoonotic and commensal bacteria whose antimicrobial resistance should be tested, and the analytical methods to be used by laboratories designated to test bacterial resistance to antibiotics (EFSA Journal, 2019).

On November 17, 2020, the European Commission decided to use the new Directive 2020/1729 for monitoring and reporting antibiotic resistance of zoonotic and commensal bacteria for the period 2021-2027 years. This Directive is in line with the recommendations of the 2019 EFSA report, as well as the procedures described by the European Committee for Antimicrobial Susceptibility Testing – EUCAST according to the international reference method ISO 20776-1: 2021 entitled "Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices - Part 1: Broth microdilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases" (Directive 2020/1729). Also, this directive authorizes the use of whole genome sequencing (WGS) techniques as an alternative to phenotypic analysis, but on a voluntary basis because a limited number of EU member states are able to use WGS for routine monitoring and analysis of antimicrobial resistance. WGS can be used in the future, but only according to the technical conditions and instructions prescribed by the European Reference laboratory for

antimicrobial resistance (EURL-AMR) in Denmark, so that the results are comparable, ie monitoring is harmonized (Directive 2020/1729).

EU member states are required to test the susceptibility of bacteria to antibiotics annually in the commensal bacteria *Escherichia coli* and *Enterococcus fecalis/fecium* and zoonotic bacteria *Salmonella* spp., and *Campylobacter jejuni/coli*, by determining the minimum inhibitory concentration of antibiotics (MIC). In addition to the listed commensal and zoonotic bacteria, EU member states are obliged to test 300 isolates of *Escherichia coli* and *Salmonella* spp. to determine whether they have the ability to produce the enzyme extended spectrum ß-lactamase (ESBL), the enzyme AmpC ß-lactamase (AmpC) and the enzyme carbapenemase (CP) (Directive 2020/1729).

Subject and scope of amr monitoring

The number of test isolates depends on the annual production of poultry, pork and beef of the monitoring country. If the annual production of broiler and pork is higher than 100,000 t, beef higher than 50,000 t and turkey higher than 10,000 t, 170 isolates of commensal and zoonotic bacteria are tested. For testing ESBL/AmpC/CP producing *Escherichia coli* and *Salmonella* spp. it is necessary to test 300 isolates annually (Table 1). EU member states that have lower annual production of poultry, pork and beef than prescribed, test 85 isolates of commensal and zoonotic bacteria, while for testing ESBL/AmpC/CP producing *Escherichia coli* and *Salmonella* spp. test 150 isolates. EU member states that failed to collect 170 isolates of commensal and zoonotic bacteria or 300 isolates of potential ESBL/AmpC/CP *Escherichia coli* and *Salmonella* spp. include in the monitoring of antimicrobial resistance the number of isolates collected that year (Directive 2020/1729).

Samples for monitoring antimicrobial resistance must be origin of healthy animal, so EU Directive 2020/1729 prescribes sampling at the slaughter line in slaughterhouses, retail outlets, border crossings, as well as on disease-free poultry farms (Directive 2020/1729). For testing the antimicrobial resistance of *Salmonella* spp. in the case of broilers, laying hens and fattening turkeys, EU member states may use isolates already obtained from the national control programs provided for in Regulation (EC) No 2160/2003 (Directive 2020/1729). Categories of animals by species, origin and number of samples are shown in Table 1. Accredited laboratories designated by the EU member state are responsible for sampling materials at slaughterhouses, retail outlets, border crossings and farms, isolation and strain identification, and for adequate transport of isolates to the National reference laboratory in designated to conduct of antimicrobial resistance testing. The National reference laboratory for antimicrobial resistance is responsible for testing the

susceptibility of the obtained bacterial isolates to antibiotics, followed by monitoring and analysis of *Escherichia coli* and *Salmonella* spp. which produce ESBL/AmpC/CP enzymes, as well as alternative methods (WGS) in ESBL/AmpC/CP producing strains of *Escherichia coli* and *Salmonella* spp. (Directive 2020/1729).

Table 1. Type and categories of animals, origin and number of isolates covered by antimicrobial resistance monitoring according to Directive 2020/1729

Bacterial species	Number of isolates	Animal species	Sample
E. coli	170	Broilers	Caecal content, fresh meat
		Fattening turkeys	Caecal content, fresh meat
		Fattening pigs	Caecal content, fresh meat
		Cattle up to 1 year of age	Caecal content, fresh meat
Salmonella spp.	170	Broilers	Caecal content, neck skin, fresh meat
		Fattening turkeys	Caecal content, neck skin, fresh meat
		Broilers, fattening turkeys, laying hens	Overshoes
		Fattening pigs	Caecal content
		Cattle up to 1 year of age	Caecal content
Campylobacter jejuni/coli	170	Broilers	Caecal content
		Fattening turkeys	Caecal content
		Fattening pigs	Caecal content
		Cattle up to 1 year of age	Caecal content
Enterococcus faecalis/fecium	170	Broilers	Caecal content
		Fattening turkeys	Caecal content
		Fattening pigs	Caecal content
		Cattle up to 1 year of age	Caecal content
ESBL/AmpC/CP produce *E. coli* and *Salomenlla spp.*	300	Broilers	Caecal content, fresh meat
		Fattening turkeys	Caecal content, fresh meat
		Fattening pigs	Caecal content, fresh meat
		Cattle up to 1 year of age	Caecal content, fresh meat

Microdilution method in broth

Broth microdilution is performed according to the reference method ISO 20776-1:2021, which determines MIC (ISO 20776-1: 2021). This method allows the tested bacterial strains to be classified as susceptible or resistant by determining the epidemiological limit value of the MIC according to EU Directive 2020/1729 (EU Directive 2020/1929). In order to harmonize the monitoring of antimicrobial resistance, EU member states are required to perform the method in sensititre Muller-Hinton broth (Thermo Scientific, United Kingdom) and sensititre plates with flat bottom (Thermo Scientific, United Kingdom) in which double-diluted antibiotics are impregnated (Table 2) (EU Directive 2020/1929). The microdilution method is intended to test pure cultures of bacteria that grow easily after overnight incubation on Columbia agar with 5% defibrinated sheep blood (ISO 20776-1: 2021). For susceptibility testing of *Escherichia coli* and *Salmonella spp*. two antibiotic panels are used, the first panel (EUVSEC3) to determine the multiresistance of the test strain (MDR) and the second panel (EUVSEC2) to determine whether the test strain has the ability to produce ESBL/AMPC/CP enzymes (Table 2). Only one panel of antibiotics is used for susceptibility testing in *Campylobacter jejuni/coli* (EUCAMP3) and *Enterococcus faecalis/fecium* (EUVENC) (Table 2) (EU Directive 2020/1729).

Table 2. Antibiotics used in monitoring of antimicrobial resistance according to EU Directive 2020/1729.

No	*Escherichia coli*	*Salmonella spp.*	*Campylobacter jejuni/coli*	*Enterococcus faecalis/fecium*
	First panel	**First panel**	**First panel**	**First panel**
	EUVSEC3	**EUVSEC3**	**EUCAMP3**	**EUVENC**
1.	Amikacin	Amikacin	Chloramphenicole	Ampicilin
2.	Ampicilin	Ampicilin	Ciprofloxacin	Chloramphenicole
3.	Azitromycin	Azitromycin	Ertapenem	Ciprofloxacin
4.	Cefotaksime	Cefotaksime	Eritromycin	Daptomicin
5.	Ceftazidime	Ceftazidime	Gentamicine	Eritromycin
6.	Choramphenicol	Choramphenicole	Tetracycline	Gentamicin
7.	Ciprofloxacin	Ciprofloxacin	/	Linezolid
8.	Colistin	Colistin	/	Quinupristin/Dalfopristin
9.	Gentamicin	Gentamicine	/	Teikopalanin
10.	Meropenem	Meropenem	/	Tetracycline
11.	Nalidixic ac.	Nalidixic ac.	/	Tigecycline
12.	Sulfametoksazole	Sulfametoksazole	/	Vankomycin
13.	Tetracycline	Tetracycline	/	/
14.	Tigecycline	Tigecycline	/	/
15.	Trimetoprim	Trimetoprim	/	/

	Second panel	Second panel	Second panel	Second panel
	EUVSEC2	**EUVSEC2**	**/**	**/**
16.	Cefepime	Cefepime	/	/
17.	Cefotaxime	Cefotaxime	/	/
18.	Cefotaxime/clav.ac.	Cefotaxime/clav.ac.	/	/
19.	Cefoxitin	Cefoxitin	/	/
20.	Ceftazidime	Ceftazidime	/	/
21.	Ceftazidime/clav.ac.	Ceftazidime/clav.ac.	/	/
22.	Ertapenem	Ertapenem	/	/
23.	Imipenem	Imipenem	/	/
24.	Meropenem	Meropenem	/	/
25.	Temocilin	Temocilin	/	/

Quality control, storage of isolates and testing for certificate

EU member states are obliged to carry out quality control in accredited laboratories for sampling, isolation and identification of bacteria, as well as in National reference laboratories designated for monitoring antimicrobial resistance according to Directive 2020/1729. Quality control includes verification of professional competence and quality control of performing methods for sampling, isolation and identification of bacteria, as well as testing for sensitivity to antibiotics. The National reference laboratory is required to store for five years at -80 ºC all isolates determined to be resistant during monitoring. In the event that EFSA or the EURL-AMR deems it necessary and relevant to confirm or further test an isolate, the National reference laboratory of an EU member state is obliged to adequately label the isolate and send it to the EURL-AMR (Directive 2020/1729).

Preparing reports and reporting

EU member states are obliged to analyze the results of one-year monitoring of antimicrobial resistance and prepare a report to the EFSA (EFSA Journal, 2021). The report should contain all relevant data on the movement and sources of zoonosis, as well as data on samples and isolates (for each isolate separately) that have been tested for antimicrobial resistance according to the instructions prescribed in Directive 2020/1729. EU member states must also describe in their reports the sampling plans, stratification and randomization procedures by animal population and food category (Directive 2020/1729). The sampling frequency was made according to the rotation model, the results are analyzed and a report is prepared for isolates originating from broilers, laying hens, broilers and fresh meat of broilers and turkeys (example 2020, 2022, 2024, 2026), and for isolates originating from fattening pigs and cattle up to one year old and fresh meat from fattening pigs and cattle up to one year old (example 2021, 2023, 2025, 2027) (Directive 2020/1729). The obtained monitoring results are reported to EFSA from 1 April to 30 May of the current year for the previous year. Upon receipt of the report from the national reference laboratories, EFSA produces a final report and mapping containing national quantitative data on antibiotic resistance

based on the results and analyzes reported by all EU member states included in the antimicrobial resistance monitoring network (EFSA Journal, 2021).

Conclusion

Continuous monitoring of bacterial resistance contributes to a better understanding of the development of resistance, its transmission between bacteria and its maintenance in nature. The food production chain is one of the main sources of pathogenic or non-pathogenic resistant bacteria for humans. Taking into account one health, EU member states have implemented monitoring of antimicrobial resistance in veterinary medicine, in order to reduce the high percentage of resistance with the rational use of antibiotics. In order to implement resistance monitoring in Republic of Serbia, it is important to establish adequate sampling strategy and collection of data on samples, as well as adequate methods of storing and sending isolates to the National reference laboratory.

Acknowledgment

The research was funded by the grant from the Ministry of Education, Science and Technological Development of the Republic of Serbia, by the Contract of implementation and fnancing of scientifc research work of NIV-NS in 2022, Contract No. 451-03-68/2022-14/200031.

Authors contributions

Writing – original draft preparation DT, MP, ĐM, DLjP, SVK and BĐ; Writing – review and editing DT and MV; Supervision – MV. All authors have read and agreed to the published version of the manuscript.

Competing interests

The authors declare that they have no competing interests

References

1. European Food Safety Authority (EFSA); European Centre for Disease Prevention and Control (ECDC). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA J*. 2021:19 (4):06490. doi:10.2903/j.efsa.2021.6490. 2. European Union. 2003. Directive 2003/99/EC of the European Parliament and of the Council on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC, 325/31 – 325/40. 3. European Union. 2013. Commission Implementing Decision 2013/652/EU of 12 November 2013 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria, 303/26 – 303/39. 4. European Food Safety Authority (EFSA); Technical specifications on harmonised monitoring ofantimicrobial resistance in zoonotic and indicator bacteriafrom food-producing animals and food. *EFSA J*. 2019;17(6):5709. doi:10.2903/j.efsa.2019.5709. 5. European Union. 2020. Commission Implementing Decision 2020/1729 of 17 November 2020 on the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria, 387/8 – 387/21. 6. EUCAST, The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 12.0, 2022. http://www.eucast.org." 7. ISO 20776-1:2021, Susceptibility testing of infectious agents and evaluation of performance of antimicrobial susceptibility test devices - Part 1: Broth micro-dilution reference method for testing the in vitro activity of antimicrobial agents against rapidly growing aerobic bacteria involved in infectious diseases (ISO 20776-1:2019, including Corrected version 2019-12).

ONE HEALTH: THE IMPORTANCE OF PRUDENT AND RESPONSIBLE USE OF ANTIMICROBIAL MEDICINES

Ena Oster[1*], Hrvoje Pavasović[1], Siniša Faraguna[2], Iva Benvin[3], Ozren Smolec[4], Frane Božić[1]

[1]*University of Zagreb, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Zagreb, Croatia*

[2]*University of Zagreb, Faculty of Veterinary Medicine, Department for Pathophysiology, Zagreb, Croatia*

[3]*University of Zagreb, Faculty of Veterinary Medicine, Clinic for Infectious Diseases, Zagreb, Croatia*

[4]*University of Zagreb, Faculty of Veterinary Medicine, Clinic for Surgery, Orthopaedics and Ophthalmology, Zagreb, Croatia*

[*]*e-mail: eoster@vef.unizg.hr*

Abstract

The extensive use of antimicrobials in human and veterinary medicine has increased the emergence and spread of antimicrobial resistance. European Centre for Disease Prevention and Control and European Medicines Agency (EMA) estimate that each year, drug-resistant infections result in at least 25 000 patient deaths and cost the EU 1,5 billion euros in healthcare costs and through loss of productivity. The first Antimicrobial Advice ad hoc Expert Group Categorisation, published in 2014., considered the risk to public health from antimicrobial resistance due to the use of antimicrobials in veterinary medicine. In 2019, EMA published updated guidelines regarding the categorisation of antimicrobials considering new experience gained and research. It categorised antimicrobial into following groups: Category A (avoid; should not be used in food-producing animals, may be given to companion animals under exceptional circumstances), Category B (restrict; critically important in human medicine, use based on antimicrobial susceptibility testing and only when there are no clinically effective antibiotics in Categories C or D), Category C (caution; should be considered only when there are no clinically effective antibiotics in Category D), and Category D (prudence; use as first line treatments, but only when medically needed). For antibiotics in all categories, unnecessary use, overly long treatment periods, and under-dosing should be avoided. Also, group treatment should be restricted to situations where individual treatment is not feasible and using an antimicrobial agent of as narrow-spectrum as possible is encouraged. Responsible, rational, and targeted use of antimicrobials should maximise the therapeutic effect and minimise the

development of antimicrobial resistance.

Key words: antimicrobial resistance, guidelines, one health

Introduction

The use of antimicrobials led to the revolution in the management of bacterial infections in human medicine. Also, antimicrobials have been used in veterinary medicine for prevention of diseases for a long time, both in production and companion animals. Successful treatment with antimicrobials is compromised by constant rise in antimicrobial resistance (AMR). It is known that AMR spreads among the environment, humans, and animals, and between similar microbes. That is why a multidisciplinary approach, such as One-health, is needed. One-Health approach is defined as "the collaborative effort of multiple health science professions, together with their related disciplines and institutions – working locally, nationally and globally – to attain optimal health for people, domestic animals, wildlife, plants and environment" (McEwen et al., 2018). Most of the classes of antimicrobials used as a treatment of human infections are also used in veterinary medicine, resulting in the spread of AMR. In the human medicine, main recommendations under One-health approach are higher consciousness while prescribing antibiotic treatment, preventing over-prescription, improvement of the hygiene conditions and infection control plans (Palma et al., 2020). Regarding the environmental sector, actions include the appropriate treatment of industrial, civil and farm waste, on the attempt to reduce the overall dissemination of the AMR between sectors (McEwen et al., 2018). In this paper, we will discuss One-Health approach regarding veterinary medicine, but first we will explain mechanisms of AMR and provide some examples of AMR that effect public health.

Mechanisms of antimicrobial resistance

There are six known mechanisms of antimicrobial resistance. Enzyme inactivation is based on the ability of bacteria to produce enzymes that break down or inactivate the antimicrobials. As an example, there are bacteria that produce β-lactamase enzymes (e.g., *Staphylococcus aureus*), which hydrolyse β-lactam ring of penicillin and cephalosporin antibiotics, and inactivate the antibiotics (Šeol et al., 2010).

Second mechanism is by reducing bacterial wall permeability. The outer membrane of some gram-negative bacteria can be a barrier to the permeability of certain antibiotics. Decreased drug intake may be due to reduced expression, alteration, or even complete loss of pores on the cell wall that otherwise allow it to

enter the bacterial cell. Changing the charge of the cell wall lipopolysaccharide from negative to neutral can prevent highly positively charged antibiotics (e.g., aminoglycosides) from passing through the outer membrane of the bacterial cell and thus reaching the cell itself (Holmes et al., 2016).

The third mechanism of AMR is by changing target site on the bacterial cell. Most antimicrobial drugs act on bacteria by specifically binding to a target site (receptor) in a bacterial cell. By changing the structure or concealing the receptor, the antibiotic cannot bind to the target site and act on the bacteria. As an example, resistance to erythromycin and clindamycin is the result of changes in the structure of the target protein of the 50S ribosome (Šeol et al., 2010).

The fourth type of AMR is by bypassing metabolic processes due to the ability of some bacteria to produce enzymes that inactivate antibiotics before entering the cytoplasm. Inactivation consists of adenylation, phosphorylation or acetylation of an antibiotic molecule. An example is streptomycin adenylation, in which enzyme streptomycin-adenyltransferase produces the inactive adenyl streptomycin compound, which has no antibacterial effect. Also, phosphorylation by phosphotransferase enzymes in the presence of ATP inactivates kanamycin.

The fifth known mechanism of AMR is by reduction of intracellular accumulation of antimicrobial drug. It is done by reducing bacterial wall permeability or increasing drug excretion by activating energy-dependent transport system. The presence of this system in the wall is determined by plasmids and chromosomes. Because the system is not specific, it is equally responsible for the resistance of gram-positive bacteria of the genus *Staphylococcus* and *Streptococcus* as well as gram-negative bacteria of the genus *Pseudomonas* and *Escherichia coli*.

In the recent times, the last mechanism of AMR has been discovered, biofilm formation. Biofilm growth has shown to preserve bacterial cells from antibiotic treatment. It is clinically relevant in the chronic infections and the potential colonization of surfaces (*e.g., Pseudomonas aeruginosa*) (Balcázar et al., 2015).

Microbial resistance to antimicrobial drugs can be innate and acquired. Acquired resistance is most important for public health. It is a result of a gene mutation, a transfer of a gene that determines resistance, or a combination of both. When fluoroquinolones are used, resistance most often occurs due to a gene mutation. Resistance is usually manifested by a slight increase in the value of the minimum inhibitory concentration (MIC) of the bacteria. The bacteria can become resistant to one antibiotic, to all members of a group of antimicrobial drugs, or to antimicrobial drugs of different groups. Also, bacteria can receive genetic material from a similar microbe in three ways: by conjugation, transduction, and transformation (Davies,

2010). Conjugation is the transfer of genetic information from a donor cell to a recipient cell via the R plasmid (e.g., enterobacteria). Transduction is the direct transfer of "naked" DNA from one bacterium to another using bacteriophages (e.g., *E. coli*). Transformation is the ability of a bacterium to take part of the donor's DNA from the environment, import it and place it in an appropriate place on the chromosome.

S. Aureus as an example of antmicrobial resistance

During World War II penicillin antibiotic were available and saved multiple lives. But soon thereafter, most isolates from hospitals in Europe were found to be resistant to penicillin. First known resistant bacteria was penicillinase-producing *Staphylococcus aureus*. These strains produced a plasmid-encoded penicillinase that hydrolyses the β-lactam ring of penicillin essential for its antimicrobial activity (Chambers et DeLeo, 2009). Penicillin-resistant strains were then observed to cause community infections; by the early 1950s and 1960s they had become pandemic. Due to resistance, other newly discovered antibiotics (primarily methicillin) were used to treat infections. In the 1960s, emergence of methicillin resistant *S. aureus* (MRSA) was noted (Barber, 1961). Although the mode of resistance was known to be different from penicillinase-mediated resistance because there was no drug inactivation, only after about 20 years it was discovered that a specific gene (mecA) encodes the low affinity penicillin binding protein (PBP 2a), which avoids the inhibitory effects of the antibiotics (Chambers et DeLeo, 2009). Unlike penicillinase-mediated resistance, which is narrow in its spectrum, methicillin resistance is broad β-lactam antibiotic class resistance to penicillins, cephalosporins, and carbapenems (Chambers et DeLeo, 2009). Outbreaks of infections caused by MRSA strains were reported in hospitals in the United States in late 1970s and by the mid-1980s were endemic (Peacock et al. 1980; Crossley et al., 1979) that continues to the present time. Due to that, hospitals started to use vancomycin, the only remaining antibiotic to which MRSA strains were susceptible. Soon after, vancomycin intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) strains of MRSA emerged (Weigel et al., 2003; Hiramatsu et al., 1997).

Pleuromutilin and colistin

Pleuromutilin is a natural antimicrobial medication produced by the fungus *Clitopilus scyphoides* (Kavanagh et al., 1951). Tiamulin and valnemulin are semi-synthetic derivates of pleuromutilin and both medications were registered and used in veterinary medicine. They are effective against gram-positive bacteria, such as

Streptococcus spp. and *Staphylococcus* spp., and they have been used for the control of respiratory and intestinal infections in different animal species, especially pigs (Jones et al., 2006). Retapamulin is the first pleuromutilin registered for human use in 2007, but only for topical use (Novak, 2011). First pleuromutilin for systemic use, approved in the USA in 2019 and in EU in 2020, is lefamulin and it is used to treat bacterial pneumonia. Both retapamulin and lefamulin are effective against MRSA. Even though pleuromutilins are unaffected by resistance to other major antibiotic classes (such as macrolides, fluoroquinolones, tetracyclines, β-lactam antibiotics, and other) and that they display slow resistance development at sub-MIC in vitro, resistant strains have been identified in human- and livestock-associated environments (Parkner et Rieldl, 2017). European Medicines Agency (EMA) recognised the potential problem of transmitting AMR from animals to humans due to several new findings (Alban et al., 2017). They had new knowledge on resistance to pleuromutilin emerging in staphylococci and enterococci, making horizontal transmission of other critical resistances such as linezolid resistance possible (Duijkeren et al., 2014). Also, potential selection of pleuromutilin-resistant staphylococci (including livestock-associated MRSA) in swine treated with pleuromutilins (EMA, 2013). There was also possible increased importance of pleuromutilins for the treatment of humans due to new product developments (retapamulin and lefamulin).

Increasing antibiotic resistance in multidrug-resistant Gram-negative bacteria (MDR-GNB) presents significant health problems worldwide, since the vital available and effective antibiotics often fail to fight MDR-GNB as well as the absence of new antibiotics (Falagas et Kasiakou, 2015). This has led to the reconsideration of old drugs as a last-hope treatment in the mid-1990s, such as colistin. Unfortunately, due to the overuse and misuse of colistin, rapid global resistance towards colistin has emerged following its resurgence, particularly *P. aeruginosa*, *A. baumannii*, and *K. pneumonia*, which are resistant against all other available antibiotics (Ahmed et al., 2020).

EMA guidelines

Due to the growing evidence of AMR and the transmission of AMR between animals and humans, there was a need for guidelines on the use of antibiotics in veterinary medicine. European Centre for Disease Prevention and Control and EMA estimate that each year, drug-resistant infections result in at least 25 000 patient deaths and cost the EU 1,5 billion euros in healthcare costs and through loss of productivity (ECDC et EMEA, 2009). The first Antimicrobial Advice ad hoc Expert Group Categorisation (AMEG), published in 2014., considered the risk to public health from

AMR due to the use of antimicrobials in veterinary medicine. Categorisation classified antimicrobials in three categories: Category 1 (antimicrobials used in veterinary medicine where risk for public health is estimated as low or limited), Category 2 (antimicrobials used in veterinary medicine where risk for public health is estimated higher) and Category 3 (not approved for use in veterinary medicine). Tang et al. (2017) later concluded in systematic review and meta-analysis that interventions that restrict antibiotic use in food-producing animals are associated with a reduction in the presence of antibiotic-resistant bacteria in these animals. A smaller body of evidence also suggested a similar association in the studied human populations, particularly those with direct exposure to food-producing animals. Furthermore, A unique aspect related to AMR and risk of resistance transfer in companion animals is their close contact with humans. This creates opportunities for interspecies transmission of resistant bacteria.

In 2019, EMA published updated guidelines regarding the categorisation of antimicrobials considering new experience gained and research. The updated criteria on which the Categorisation is based are as follows:

1. If the (sub)class or group is authorised for use as a veterinary medicine in the EU
2. The importance of the (sub)class or group to human medicine according to the WHO ranking and considering the EU situation
3. The knowledge of factors influencing the likelihood and consequences of AMR transfer from animals to humans, in particular considering mechanisms where a single gene confers multiresistance (or resistance to several classes)
4. The availability of alternative antibiotic (sub)classes in veterinary medicine with lower AMR risk to animal and public health.

Thus, EMA categorised antimicrobial into following groups (as shown in Table 1):

1. Category A (avoid): antibiotics in this category are not authorised in veterinary medicine in the EU, should not be used in food-producing animals, and may be given to companion animals under exceptional circumstances.
2. Category B (restrict): antibiotics in this category are critically important in human medicine and use in animals should be restricted to mitigate the risk to public health. Use should be based on antimicrobial susceptibility testing and only when there are no clinically effective antibiotics in Categories C or D.
3. Category C (caution): for antibiotics in this category there are alternatives in human medicine. They should be considered only when there are no clinically effective antibiotics in Category D)

4. Category D (prudence): antibiotics in this category should be used as first line treatments, but only when medically needed

Table 1 Adjusted summary of AMEG Categorisation (EMA, 2019)

AMEG Categories	Antibiotic class, subclass	Example of antibiotics
Category A (Avoid)	Amdinopenicillins	mecillinam, pivmecillinam
	Carbapenems	meropenem, doripenem
	Other cephalosporins** and penems (ATC code J01DI), including combinations of 3rd-generation cephalosporins with β-lactamase inhibitors	ceftobiprole, ceftaroline, ceftolozane-tazobactam, faropenem
	Glycopeptides	vancomycin
	Glycylcyclines	Tigecycline
	Ketolides	telithromycin
	Lipopeptides	daptomycin
	Monobactams	Aztreonam
	Oxazolidinones	Linezolid
	Penicillins: carboxypenicillins and ureidopenicillins, including combinations with β-lactamase inhibitors	piperacillin-tazobactam
	Phosphonic acid derivates	Fosfomycin
	Pseudomonic acids	Mupirocin
	Rifamycins (except rifaximin)	Rifampicin
	Riminofenazines	clofazimine
	Streptogramins	pristinamycin, virginiamycin
	Sulfones	Dapsone
	Drugs used solely to treat tuberculosis or other mycobacterial diseases	isoniazid, ethambutol, pyrazinamide, ethionamide
	Substances newly authorised in human medicine following publication of the AMEG categorisation.	To be determined
Category B (Restrict)	Cephalosporins: 3rd- and 4th-generation, except combinations with β-lactamase inhibitors	ceftiofur, cefovecin, cefquinome
	Polymyxins	colistin, polymyxin B
	Quinolones: fluoroquinolones and other quinolones	enrofloxacin, ciprofloxacin, ofloxacin, oxolinic acid
Category C (Caution)	Aminoglycosides (except spectinomycin)	streptomycin, gentamicin
	Aminopenicillins in combination	amoxicillin-clavulanic acid

Category C (Caution)	with β-lactamase inhibitors	
	Amphenicols	florfenicol, thiamphenicol
	Cephalosporins: 1st- and 2nd-generation, and cephamycins	cefalexin, cefapirin
	Macrolides (not including ketolides)	tylosin, tulathromycin
	Lincosamides	clindamycin, lincomycin
	Pleuromutilins	tiamulin, valnemulin
	Rifamycins: rifaximin only	rifaximin
Category D (Prudence)	Aminopenicillins, without β-lactamase inhibitors	amoxicillin, ampicillin
	Cyclic polypeptides	bacitracin
	Nitrofuran derivatives*	furazolidone
	Nitroimidazoles*	metronidazole
	Penicillins: Anti-staphylococcal penicillins (β-lactamase-resistant penicillins)	cloxacillin
	Penicillins: Natural, narrow spectrum penicillin (β-lactamase-sensitive penicillins)	benzylpenicillin, phenoxymethylpenicillin
	Aminoglycosides: spectinomycin only	spectinomycin
	Steroid antibacterials*	fusidic acid
	Sulfonamides, dihydrofolate reductase inhibitors and combinations	sulfadiazine, trimethoprim
	Tetracyclines	oxytetracycline, doxycycline

*Authorised for companion animals only
**Other than 1st-, 2nd-, 3rd- and 4th-generation

For antibiotics in all categories, unnecessary use, overly long treatment periods, and under-dosing should be avoided. Also, group treatment should be restricted to situations where individual treatment is not feasible and using an antimicrobial agent of as narrow-spectrum as possible is encouraged. Responsible, rational, and targeted use of antimicrobials should maximise the therapeutic effect and minimise the development of AMR.

Acknowledgement
The participation in the conference "Antimicrobial resistance in veterinary medicine - current state and perspectives" is supported by COST Action 18217 European Network for Optimization of Veterinary Antimicrobial Treatment

References

1. Aarestrup F.M., Wegener H.C., Collignon P. 2008. Resistance in bacteria of the food chain: Epidemiology and control strategies. Expert Rev. Anti. Infect. Ther., 6:733–750. doi: 10.1586/14787210.6.5.733. 2. Alban L., Ellis-Iversen J., Andreasen M., Dahl J., Sönksen U.W. 2017. Assessment of the Risk to Public Health due to Use of Antimicrobials in Pigs-An Example of Pleuromutilins in Denmark. Front Vet Sci., 4:74. doi:10.3389/fvets.2017.00074 3. Ahmed E.-S., Zhong L.L., Shen C., Yang Y., Doi Y., Tian G.B. 2020. Colistin and its role in the Era of antibiotic resistance: an extended review (2000-2019). Emerg Microbes Infect., 9(1):868-885. doi:10.1080/22221751.2020.1754133 4. Balcázar J.L., Subirats J., Borrego C.M. 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol., 6:1216. doi: 10.3389/fmicb.2015.01216. 5. Barber M. 1961. Methicillin-resistant staphylococci. J Clin Pathol., 14:385–93. 6. Chambers H.F., Deleo F.R. 2009. Waves of resistance: Staphylococcus aureus in the antibiotic era. Nat Rev Microbiol., 7(9):629-641. doi:10.1038/nrmicro2200 7. Crossley K., Landesman B., Zaske D. 1979. An outbreak of infections caused by strains of Staphylococcus aureus resistant to methicillin and aminoglycosides. II. Epidemiologic studies. J Infect Dis., 139:280–7. 8. Davies J., Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev., 74(3):417-433. doi:10.1128/MMBR.00016-10 9. ECDC/EMEA. 2009 Joint Technical Report: The bacterial challenge: time to react. doi: 10.2900/2518 10. European Medicines Agency. 2013. Reflection Paper on Use of Pleuromutilins in Food-Producing Animals in the European Union: Development of Resistance and Impact on Human and Animal Health. EMA/CVMP/AWP/119489/2012. 11. Falagas M.E., Kasiakou S.K. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. Clin Infect Dis., 40(9):1333–1341. doi: 10.1086/429323 12. Hiramatsu K., Aritaka N., Hanaki H., Kawasaki S., Hosoda Y., Hori S., Fukuchi Y., Kobayashi I. 1997. Dissemination in Japanese hospitals of strains of Staphylococcus aureus heterogeneously resistant to vancomycin. The Lancet, 350, 1670-1673. 13. Holmes A.H., Moore L.S., Sundsfjord A., Steinbakk M., Regmi S., Karkey A., Guerin P.J., Piddock L.J. 2016. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet, 9;387(10014):176-87. doi: 10.1016/S0140-6736(15)00473-0 14. Jevons M. 1961. "Celbenin"-resistant staphylococci. Br Med J., 1:124–5 15. Jones R.N., Fritsche T.R., Sader H.S. 2006. Activity of retapamulin (SB-275833), a novel pleuromutilin, against selected resistant gram-positive cocci. Antimicrob Agents Chemother, 50: 2583 –6. doi: 10.1128/AAC.01432-05. 16. Kavanagh F., Hervey A, Robbins W.J. 1951. Antibiotic substances from Basidiomycetes: VIII. Pleurotus multilus (Fr.) Sacc. and Pleurotus passeckerianus Pilat. Proc Natl Acad Sci USA, 37: 570–4 17. McEwen S.A., Collignon P.J. 2018. Antimicrobial Resistance: A One Health Perspective. Antimicrob. Resist. Bact. Livest. Companion Anim., 6:521–547. doi: 10.1128/microbiolspec.ARBA-0009-2017 18. Novak R. 2011. Are pleuromutilin antibiotics finally fit for human use? Ann NY Acad Sci 2011; 1241: 71 –81. doi: 10.1111/j.1749-6632.2011.06219.x. 19. Palma E., Tilocca B., Roncada P. 2020. Antimicrobial Resistance in Veterinary Medicine: An Overview. Int J Mol Sci., 21(6):1914. doi:10.3390/ijms21061914 20. Paukner S., Riedl R. 2017. Pleuromutilins: Potent Drugs for Resistant Bugs-Mode of Action and Resistance. Cold Spring Harb Perspect Med., 7(1):a027110. doi:10.1101/cshperspect.a027110 21. Peacock J.E., Jr, Marsik F.J., Wenzel R.P. 1980. Methicillin-resistant Staphylococcus aureus: introduction and spread within a hospital. Ann Intern Med., 93:526–32. 22. Roundtree P., Freeman V. 1956. Infections caused by a particular phage type of Staphylococcus aureus. Med J Aust., 42:157–61. 23. Šeol B., Matanović K., Terzić S. 2010. Rezistencija bakterija na antimikrobne lijekove. In Antimikrobna terapija u veterinarskoj medicini. Ed. V. Herak-Perković, Medicinska Naklada, Zagreb, pp. 33-41. 24. Tang K.L., Caffrey N.P., Nóbrega D.B., Cork S.C., Ronksley P.E., Barkema H.W., Polachek A.J., Ganshorn H., Sharma N., Kellner J.D., Ghali W.A. 2017. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet Health, 1(8):e316-e327. doi: 10.1016/S2542-5196(17)30141-9. 25. van Duijkeren E., Greko C., Pringle M., Baptiste K.E., Catry B., Jukes H., Moreno M.A., Ferreira Pomba M.C.A., Pyorala S., Rantala M., Ruzauskas M., Sanders P., Teale C., Threlfall E.J., Torren-Edo J., Torneke K. 2014. Pleuromutilins: use in food-producing animals in the European Union, development of resistance and impact on human and animal health, J Antimicrob Chemother, 69: 2022 –2031. doi:10.1093/jac/dku123. 26.Weigel L.M., Clewell D.B., Gill S.R., Clark N.C., McDougal L.K., Flannagan S.E., Kolonay J.F., Shetty J., Killgore G.E., Tenover F.C. 2003. Genetic analysis of a high-level vancomycin-resistant isolate of Staphylococcus aureus. Science, 302(5650):1569-71. doi: 10.1126/science.1090956.

MAGNETIC RESONANCE IMAGING IN MEASUREMENT OF ANTIMICROBIAL RESISTANCE EFFECT

Ömer Gürkan Dilek[1*]

[1]*Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Anatomy, Burdur, Turkey*

[*]*e-mail: ogdilek@mehmetakif.edu.tr*

Abstract

Antimicrobial resistance is a growing problem in veterinary and human medicine in Europe and the rest of the world. Antimicrobials are essential for the medical care and health of companion animals and livestock, but inappropriate use of antimicrobials (such as use in an untargeted manner, at sub-therapeutic doses, repeatedly, or for inappropriate periods) can result in the development of antimicrobial resistance. Antimicrobials used in animals are closely related to those used in human medicine. Magnetic Resonance Imaging (MRI) is a non-invasive imaging technology that produces three-dimensional detailed anatomical images. It is often used for disease detection, diagnosis, and treatment monitoring. It is based on sophisticated technology that excites and detects the change in the direction of the rotational axis of protons found in the water that makes up living tissues. With the advancement in nanoscience and nanotechnology, promising opportunities for examining the bacterial effect of metal nanoparticles were demonstrated in the literature. Gadolinium is one of the antimicrobials and it's often used as an antimicrobial surrogate. In the literature, the effect of dose and distribution of Gadolinium is measured by using MRI. Magnetic resonance imaging shows local delivery throughout surgical wounds. In the future, MRI can be used to determine the different antimicrobial agent doses and antimicrobial resistance.

Key words: antibiotic resistance, nanoscience, magnetic resonance imaging

Antimicrobial resistance

The discovery of antimicrobial drugs first began by Pasteur and Joubert by determining that anthrax bacilli that can grow in sterile urine cannot grow in urine contaminated with other bacteria. In 1909, German bacteriologist Paul Ehrlich conducted research on the treatment of infection and found that an arsenic-based substance was effective in the early stages of syphilis, however, he introduced the concept of "selective toxic effect". The selective toxic effect refers to substances that harm microorganisms when chemotherapeutic agents are used in low concentrations. Alexander Fleming observed in 1928 that staphylococci did not grow around the mold fungus he had grown in culture in the laboratory. Later, he discovered that the filtrate he obtained from these fungi was effective against many bacteria, and the discovery of antimicrobial drugs was made by Sir Alexander Fleming with penicillin. Alexander

Fleming, with his discovery of penicillin, was awarded the Nobel Prize in 1945 and said that bacteria can gain resistance when exposed to these substances for a certain period (Kayış, 2019; Abbasoğlu and Çevikbaş, 2011).

Resistance to antimicrobial drugs may be different in microorganisms. A microorganism can develop more than one mechanism of resistance to the same antimicrobial drug. Developing resistance in this way is called 'cross-resistance'. The development of resistance of microorganisms to many antimicrobial drugs with different structures and mechanisms of action is called 'multi-drug resistance'. The problem of resistance caused by Gram-negative microorganisms that have developed since the 1970s has become very important today. The problem of resistant bacteria occurs mostly in hospitals; because hospitals are the places where the use of antimicrobial drugs is the highest. Microorganisms with the most common resistance problem in nosocomial infections in Turkey; *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Coagulase Negative Staphylococci*, *Enterobacter* spp., *Enterococci*, *Pseudomonas aeruginosa* and *Acinetobacter* spp. (Kayış, 2019; Abbasoğlu and Çevikbaş, 2011).

The resistance mechanisms of bacteria against antimicrobial drugs can be grouped under the following headings:

1. Using the alternative metabolic pathway: Unlike target changes in some bacteria, a new metabolic pathway can be developed that will eliminate the need for a drug-sensitive target. For example, the combination of trimethoprim/sulfamethoxazole inhibits enzymes that play a role in the replication of the chromosome, which is vital for bacteria. Instead of synthesizing folate, bacteria use an alternative metabolic pathway by taking ready-made folate from the environment (Kayış, 2019; Işık 2008).

2. Change in drug target: Target site changes usually result from mutation of the bacterial gene on the chromosome and selection in the presence of the antimicrobial drug. Resistance to rifamycin occurs as a result of a target point mutation in RNA polymerase, and to quinolones as a result of target point mutation that occurs in DNA gyrase mutations. Many of the mutations responsible for quinolone resistance are seen in the genes encoding the Gyrase enzyme. The most known chromosomal mutations today are those occurring in the gyrA subunit (Kayış, 2019).

3. Excretion of the drug by active pumping: The proteins of the active pumping system in bacteria are proteins that regulate the intake of nutrients and ions into the cell, the removal of metabolic end-products and harmful substances from the cell, and the relations of bacteria with each other and their environment. The efflux pump proteins are proteins that, when synthesized at the structural level, contribute to the bacteria's natural resistance. Their high level of synthesis, on the other hand, leads to the formation of high-level multi-drug resistance in one step against many compounds, including many antimicrobial drugs, disinfectants, and dyes (Kayış, 2019; Coyne et al., 2011).

Resistance to antimicrobial drugs can take various forms:

1. Resistance Due to Environment and Conditions: Antimicrobial drugs that respond to in vitro tests may not respond in-vivo due to changes in oxygen pressure, pH changes in the tissue, and the inability of the antimicrobial drug to reach the infection site.

2. Natural Resistance: Some antimicrobial drugs do not affect the bacteria due to the genetic characteristics of the bacteria; This feature is defined as the natural resistance of the bacteria.

3. Acquired Resistance: Changes in genetic traits in bacteria occur through mutations in transposon or plasmid DNA. It is the resistance that occurs by transferring conjugation, transformation, or transduction by resistant bacteria (Kayış, 2019).

Magnetic resonance imaging (mri)

Magnetic resonance (MR) imaging is a method mostly used in medicine for imaging the internal structure of living things. Living tissue with a high level of magnetism is displayed by the projection method. Due to its different characteristics, computerized tomography can also be supported in the detection of diseases. This procedure, generally known as MRI, is nuclear magnetic resonance imaging. It creates an image according to the densities and movements of the hydrogen atoms in the tissue. No radiation is used in MR, instead, protons in the nuclei of hydrogen atoms in the body are excited by a magnetic field. Signals reaching the receivers are converted into black and white images (results can be colored in perfusion imaging) by computer analysis. The magnetic field used for this purpose is in the range of 1 - 1.5 Tesla. For comparison, the earth's magnetic field (the magnetic field that points the needle of a compass north) is at the order of 0.5 Gauss. 1 Tesla is equal to 10,000 Gauss. Therefore, a magnetic field about 25 thousand times the magnetic field strength of the earth is used in the MR device. This very strong magnetic field works under control. All images are created in the digital environment and are very different from other imaging methods. Today, MR is used especially for imaging soft tissues. It is frequently used in the diagnosis of central nervous system (brain and spinal cord) diseases, sports injuries, musculoskeletal system, especially meniscus, and lumbar hernia, as well as the evaluation of all kinds of neurological, abdominal (Figure 1), and osteological diseases (D'Anjou, 2018).

The main requirement for the use of MRI is that it is the imaging modality with the highest resolution of soft-tissue contrast. It is easier to distinguish the soft tissues that make up the majority of the body under high contrast resolution and to reveal their internal structures better. With MRI, multi-plane images can be obtained without changing the patient's position. Since there is no risk of ionizing radiation. It can be repeated many times in the same patient. As mentioned above, not only anatomical details are given with MRI, but also functional imaging (such as MR-Spectroscopy, Diffusion-Perfusion imaging, and activation) can be performed. (Frahm et al., 1992).

The duration of magnetic resonance imaging varies according to the region examined, the number of regions, and the pre-diagnosis and takes 15 minutes. with 75 min. It may take between In addition, if necessary, contrast shots are taken by using contrast material via IV (intravenous) during the examination (D'Anjou, 2018).

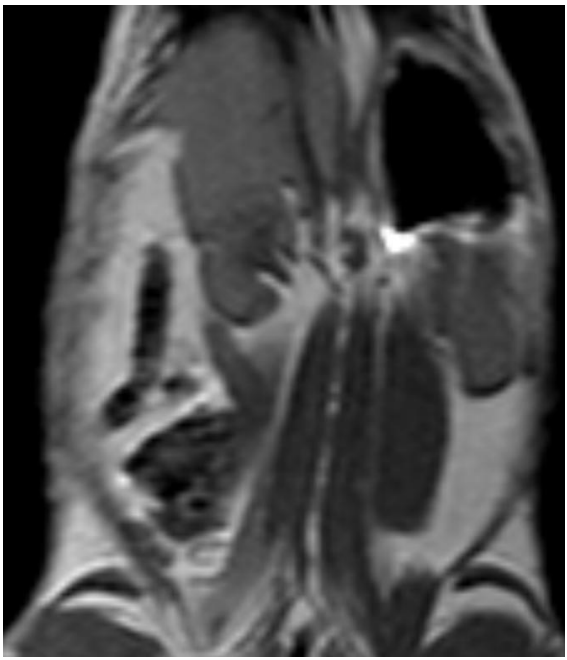


Figure 1: Coronal T1 weighted MRI image

Magnetic resonance imaging to determine antimicrobial effect

Magnetic resonance imaging was used for medical imaging and to diagnose illnesses however nowadays it's changing. Today, Gadolinium is a nanoparticle used for antimicrobial surrogates on rabbits for the wound model. The antimicrobial effect of nanoparticles' local tissue distribution on muscles is calculated by using magnetic resonance imaging (McLaren et al., 2014; Nyenhuis and Duan 2009). On the other hand, the antimicrobial effect and resistance to pyrogenic vertebral osteomyelitis were assessed by using magnetic resonance imaging (Jeon et al., 2020). New studies show that MRI will play an active role in monitoring antimicrobial resistance. In the future, MRI can be used to determine the different antimicrobial agent doses and antimicrobial resistance.

Acknowledgments

The participation in the conference "Antimicrobial resistance in veterinary medicine – current state and perspectives" is supported by COST Action 18217 European Network for Optimization of Veterinary Antimicrobial Treatment

References

1. Abbasoğlu U., Çevikbaş, A. 2011. Farmasötik Mikrobiyoloji. 1. baskı. Efil Yayınevi, Ankara, pp. 527-531. 2. Coyne S., Courvalin P., Pe´richon B. 2011. Efflux-Mediated Antibiotic Resistance in Acinetobacter spp. Antimicrobial Agents and Chemotherapy, 55(3): 947-953. 3. D'Anjou M. A. 2018. Principles of computed tomography and magnetic resonance imaging. In Textbook of veterinary diagnostic radiology. (Seventh edition) St. Louis, Missouri, pp.72. 4. Frahm J., Gyngell M. L., Hanicke W. 1992. Rapid scan techniques. In: Magnetic Resonance Imaging. Stark DD, Bradley WG. eds. Second ed. Mosby year book St. Louis, pp 165. 5. Işık Y. 2008. Pseudomonas aeruginosa Kökenlerinde Kinolon Direncinin Moleküler Olarak Saptanması, Yüksek Lisans Tezi, Ankara: Gazi Üniversitesi, pp:26. 6. Jeon I., Kong E., Yu D., Hong C. P. 2020. Clinical and radiological analysis of pyogenic vertebral osteomyelitis immediately after successful antimicrobial therapy: considerations for assessing therapeutic response. Diagnostics, 10(11): 861. 7. Kayış A. 2019. Antimicrobial Resistance Mechanisms. Aydın Sağlık Dergisi, 5(1):1-12. 8. McLaren, A., Giers, M. B., Fraser, J., Hosack, L., Caplan, M. R., McLemore, R. 2014. Antimicrobial Distribution From Local Delivery Depends on Dose. Clinical Orthopaedics and Related Research, 472(11), 3324–3329. 9. Nyenhuis, J., Duan, L. 2009. An Evaluation of MRI Safety and Compatibility of a Silver-Impregnated Antimicrobial Wound Dressing. Journal of the American College of Radiology, 6(7):500–505.

SIGNIFICANCE OF ANTIBIOTIC RESIDUES IN MILK

Jovan Stanojević[1*], Miodrag Radinović[1], Tijana Kukurić[1],

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: jovan2912@email.com*

Abstract

Responsible use of antimicrobial drugs today is one of the imperatives in both human and veterinary medicine. The treatment of many diseases requires the use of antibiotics and it's still indispensable in the treatment of various infections. Although antibiotics are often used in medicine and veterinary medicine, it need used proper. It included antibiogram and the correct therapeutic protocol. Inadequate choice of antibiotics as well as subdosing lead to the development of resistance genes in bacteria. These resistance bacteria can endanger human health. Residues of antibiotics in milk and other animal products can also be harmful to humans, including allergic reactions, immunopathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity and even anaphylactic shock in humans. For this reason, the rational and correct use of antibiotics in veterinary medicine is very important. That mean antibiotics should be use only after make antibiogram and isolation bacteria. It is also important that milk during withdrawal period of antibiotics is not used in the diet of humans or other animals. Such milk should be safely destroy while there are antibiotics residue above the permitted levels.

Key word: antibiotic residues, milk, resistence, human health

Sažetak

Odgovorna upotreba antimikrobnih lekova danas je jedan od imperativa kako u humanoj tako i u veterinarskoj medicini. Lečenje mnogih bolesti zahteva upotrebu antibiotika i oni su još uvek neophodni u lečenju različitih infekcija. Iako se antibiotici često koriste kako u humanoj, tako i u veterinarskoj medicini, potrebno ih je pravilno koristiti. To uključuje antibiogram i pravilan terapijski protokol. Neadekvatan izbor antibiotika kao i subdoziranje dovode do razvoja gena rezistencije kod bakterija. Ove rezistentne bakterije mogu ugroziti i ljudsko zdravlje. Rezidue antibiotika u mleku i drugim životinjskim proizvodima takođe mogu biti štetni za ljude, uključujući: alergijske reakcije, imunotoksičnost, kancerogenost, mutagenost, nefrotoksičnost, hepatotoksičnost, reproduktivne poremećaje, toksičnost koštane srži, pa čak i

anafilaktički šok kod ljudi. Iz tog razloga je veoma važna racionalna i pravilna upotreba antibiotika u veterini. To znači da antibiotike treba koristiti tek nakon izrade antibiograma i izolacije bakterija. Takođe je važno da se mleko tokom karence antibiotika ne koristi u ishrani ljudi ili drugih životinja. Takvo mleko treba neškodljivo ukloniti sve dok sadrži rezidue antibiotika iznad dozvoljenih granica.
Ključne reči: rezidue antibiotika, mleko, rezistencija, zdravlje ljudi

Introduction

Nowadays, various types of antibiotics are being used worldwide in veterinary treatment of the livestock. Significant portions of antibiotics are released through milk of dairy animals unaltered and exert serious harmful effects on human health. Every year, 63,151 ± 1,560 tons of antibiotics are being used in livestock worldwide (Van Boeckel et al., 2015). Due to some positive impacts, multiple veterinary antibiotics have been used worldwide recently for promoting growth and treatment of the livestock (Sabbya et al., 2019; Arikan, 2008). Residues of these antibiotics can be accumulated in dairy cattle, in addition to promoting contamination of the environment and, in more serious cases, in milk, causing a public health problem. Milk is an important and essential food for humans because of its richness in nutrients. It is highly valuable and consumed largely by people of all ages throughout the world. Milk is a perishable commodity susceptible for contamination with various chemical contaminants including Anthelmintic drugs, chlorinated pesticides, organophosphates, herbicides, fungicides, antibiotics, hormones and disinfectants. Antibiotic contamination of milk poses significant threat to safety of milk and is regarded as global public health hazard (Kumarswamy et al., 2018). Different regulatory agencies establish maximum residue limits for these antibiotics in milk, so it becomes important to develop sensitive analytical methods for monitoring these compounds (Lucas et al., 2021).

Antibiotics

Antibiotics are defined as the substances that are able to kill or inhibit the growth of various microorganisms. They are produced naturally by living organisms or synthetically in laboratory conditions. Antibiotics are administered to animals in different ways: oral, parenteral or topical. It is known that residue levels occurring from injectable sources are more than those resulting from feeding (Katz and Brady, 2000). The main use of antibiotics in animals is for the treatment and prevention of diseases including mastitis, arthritis, respiratory diseases, gastrointestinal and other bacterial infections (Darwish et al., 2013). The most usage of antibiotic in dry cows is

treatment of mastitis. This treatment can be local in mammary gland, or systemic like parenteral application including intramuscular, intravenosus or subcutaneous application.

Antibiotics residues

European Union (EU) defines residues as pharmacologically active substances (whether active principles, recipients, or degradation products) and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal products in question has been administered (Sabbya et al., 2019). The administered parent antibiotics or their metabolites become deposited in animal tissues and matrix intended to be used for human consumption, where the concentration is beyond the permitted level for a certain period of time, known as antibiotics residues. Antibiotic usage in animals may result antibiotic residues in foodstuffs such as milk, egg and meat.

Maximum residue limit (MRL)

Maximum level or concentration of a drug or chemical thought to be non-hazardous and permitted by the regulatory bodies in or on food or feed intended to be used for animal or human consumption at a specified point of time, known as MRL. The unit used for this maximum allowable concentration is milligrams per kilogram of solid products and milligrams per liter for liquids (Kebede et al., 2014). Milk and milk products contaminated with antibiotic residue above maximum residue limit (MRL) are considered unfit for human consumption. Hence, it is necessary to routinely screen milk samples for antibiotic residues (Kumarswamy et al., 2018).

Effect of antibiotic residues to human health

Antibiotics may enter to human body in different ways as directly or indirectly by using them in treatment disease or to enter low doses of antibiotic by foodstufs known as antibiotic residues (Phillips et al., 2004). Antibiotic usage in animals may leave antibiotic residues in foodstuffs such as milk, egg and meat. These residues may cause various toxic effects such as transfer of antibiotic resistant bacteria to humans, allergy, immunopathological effects, carcinogenicity (sulphamethazine, oxytetracycline, furazolidone), mutagenicity, nephropathy (gentamicin), hepatotoxicity, reproductive disorders, bone marrow toxicity (chloramphenicol) and even anaphylactic shock in humans (Darwish et al., 2013; Nisha, 2008). One of the most important adverse effects of antibiotics in food is allergic reactions. Many of the drugs and antibiotics can elicit allergic reactions. The majority of information is

related to hypersensitivity of penicillin, aminoglycosides and tetracyclines (Katz and Brady, 2000). The most important adverse effect of antibiotic residues is the transfer or antibiotic resistant bacteria to the humans due to the mobile properties of resistance. More people die from antibiotic resistant bacterial infections. It is estimated that by the year 2050, antibiotic resistant microorganisms will kill more people than cancer (Levy and Marshall, 2004).

Withdrawal time

This term is often used more broadly to describe the time needed after drug administration to any food animal where below a determined MRL may be found in marketed meats, eggs, organs, or other edible products. The withdrawal time may vary largely depending on chemical and physical properties of drugs and route of administration (Beyene, 2016).

Conclusion

Antibiotic residues in milk have a negative effect on the health of humans and other animals. Antibiotic residues also reach the environment and lead to the development of resistant bacteria. Reduction of antimicrobial resistance can be achieved only by rational and proper use of antibiotics in veterinary medicine. That mean antibiotics should be use only after make antibiogram and isolation bacteria. Harmful effects of antibiotic residues in food stufs on human health can be prevented by discarding and destroying animal products containing antibiotic residues above the permitted level (MRL).

References

1. Arikan OA. (2008) The fate of chlortetracycline during the anaerobic digestion of manure from medicated calves. J Hazard Mater. 2008;158(2–3):485–90. https://doi.org/10.1016/j.jhazmat.2008.01.096. 2. Beyene T. (2016) Veterinary drug residues in food-animal products: its risk factors and potential effects on public health. http://dx.doi.org/10.4172/2157-7579.1000285 J Vet Sci Technol;7(1):1–7. 3. Darwish, W. S., Eldaly, E. A., El-Abbasy, M. T., Ikenaka, Y., Nakayama, S., & Ishizuka, M. (2013). Antibiotic residues in food: the African scenario. Japanese Journal of Veterinary Research, 61(Supplement), S13-S22. 4. Katz, S. E., & Brady, M. S. (2000). Antibiotic residues in food and their significance. Food Biotechnology, 14(3), 147-171. 5. Kebede G, Zenebe T, Disassa H, Tolosa T. (2014) Review on detection of antimicrobial residues in raw bulk milk in dairy farms. Afr J Basic Appl Sci.;6(4):87–97. 6. Kumarswamy, N., Latha, C., Vrinda, K. M., Sethukekshmi, C., & Mercy, K. A. (2018). Detection of antibiotic residues in raw cow milk in Thrissur, India. Pharma Innov J, 7(8), 452-4. 7. Levy, S.B., Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10, S122 8. Lucas Vinícius de Faria, Thalles Pedrosa Lisboa, Náira da Silva Campos, Guilherme Figueira Alves, Maria Auxiliadora Costa Matos, Renato Camargo Matos, Rodrigo Alejandro Abarza Munoz, Electrochemical methods for the determination of antibiotic residues in milk: A critical review, Analytica Chimica Acta, Volume 1173, 2021, 338569, ISSN 0003-2670, https://doi.org/10.1016/j.aca.2021.338569. 9. Nisha, A., (2008) Antibiotic residues-a global health hazard. Vet. World 1, 375. 10. Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., Waddell, J., 2004. Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. J. Antimicrob. Chemother. 53, 28–52. 11. Sachi, S., Ferdous, J., Sikder, M. H., & Azizul Karim Hussani, S. M. (2019). Antibiotic residues in milk: Past, present, and future. Journal of advanced veterinary and animal research, 6(3), 315–332. https://doi.org/10.5455/javar.2019.f350 12. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. (2015) Global trends in antimicrobial use in food animals. Proc Natl Acad Sci USA. 112(18):5649–54. https://doi.org/10.1073/pnas.1503141112

ANTIMICROBIAL RESISTANCE IN HORSES

Tijana Kukurić[1*], Mihajlo Erdeljan[1], Jovan Stanojević[1], Annamaria Galfi Vukomanović[1]

[1]*University of Novi Sad, Faculty of Agriculture, Department of Veterinary Medicine, Novi Sad, Serbia*

[*]*e-mail: tijana.kukuric@gmail.com*

Abstract

Antimicrobial resistance is an increasingly recognized global public health threat to the modern health-care system. A group of bacteria under an acronym ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, P. aeruginosa* and *Enterobacter* spp.), for which the therapeutic options are increasingly limited, represent a significant problem, in both human and veterinary medicine. That is why "one health" approach appears to be a winning strategy to combat and reduce the burden of antimicrobial resistance, but it requires combined forces and resources that are consistently and effectively implemented by both human and veterinary health professionals. The aim of this paper is to present current state of antimicrobial resistance in horses, to hightlight bacterial strains of importance, as well as to refer veterinarians to the current situation in the fight against antimicrobial resistance.

Key words: Antimicrobial resistance, AMR, veterinary medicine, horse

Sažetak

Antimikrobna rezistencija je sve više priznata globalna pretnja po javno zdravlje i savremeni zdravstveni sistem. Grupa bakterija pod akronimom ESKAPE (*Enterococcus faecium, Staphilococcus aureus, Klebsiella pneumoniae, A. baumannii, P. aeruginosa* i *Enterobacter* spp.), za koje su terapijske mogućnosti sve ograničenije, predstavljaju značajan problem, kako u humanoj tako i u veterinarskoj medicini. Iz tog razloga pristup „jedno zdravlje" jeste pobednička strategija za borbu protiv rezistencije na antimikrobne lekove, ali zahteva kombinovane snage i resurse koji se dosledno i efikasno primenjuju, kako humanih tako i veterinarskih zdravstvenih radnika. Cilj ovog rada je da prikaže trenutno stanje i problematiku antimikrobne rezistencije kod konja, da se istaknu sojevi bakterija od značaja, kao i edukacija veterinara o aktuelnoj situaciju u borbi protiv rezistencije na antimikrobne lekove.

Ključne reči: antimikrobna rezistencija, AMR, veterinarska medicina, konj

Introduction

Antimicrobial resistance (AMR) is an increasingly recognized global public health threat to the modern health-care system, that could hamper the control and treatment of infectious diseases (Steinman and Navon-Venezia, 2020). It represents a significant challenge for both human and veterinary medicine (Vincze et al., 2014). While acknowledged as an emerging problem in companion animals, the carriage of antimicrobial resistance bacteria by horses has only comparatively recently started to receive attention (Maddox et al., 2015).

When a horse with a suspected bacterial infection is presented to a veterinarian, the ideal thing to do will be to obtain appropriate and correctly collected samples for culture and antimicrobial susceptibility testing, and make therapeutic decisions based on the laboratory results (Johns and Adams, 2015). However, this is not practical in most ambulatory and hospital clinical situations; for example, in life-threatening bacterial infections where waiting for laboratory results could potentially affect the clinical outcome, as well as the long-term performance, of the animal (Johns and Adams, 2015; Hughes et al., 2013). Faced with the above challenges, most veterinarians will resort to empirical antimicrobial prescribing based on anticipated bacterial isolates and susceptibility patterns (Hughes et al., 2013); knowledge gained from past experience or what they learned from their clinical years at school (Chipangura et al., 2017). Since empirical antimicrobial prescribing is common practice, trends in AMR from recent clinical cases need to be available to guide empirical selection practice, especially if sensitivity testing is not possible. On-going monitoring of resistance is vital to ensure that empirical antimicrobial therapy is evidence-based and current (Toombs-Ruane et al., 2015; Bowen, 2013).

Antimicrobial resistant trends, when relevant for a particular area, will allow veterinarians to make informed decisions regarding appropriate antimicrobial choice awaiting results from culture and susceptibility testing (Johns and Adams, 2015). Since continuous antimicrobial usage is considered the biggest driver for AMR development, identifying AMR trends can be used in making decisions to limit the use of particular antimicrobials thereby minimising the progression of resistance (Bowen, 2013).

Much research has focused on methicillin-resistant Staphylococcus aureus (MRSA), but clinically significant antimicrobial resistance is encountered in many other bacterial species, notably in Gram-negative members of the Enterobacteriaceae, such as extended-spectrum beta-lactamases (ESBL)-producing Escherichia coli and multidrug-resistant (MDR) Salmonella (Steinman and Navon-Venezia, 2020; Maddox et al., 2015).

The aim of this paper is to present current state of antimicrobial resistance in horses, to hightlight bacterial strains of importance, as well as to refer veterinarians to the current situation in the fight against antimicrobial resistance.

Staphylococcal species

Staphylococcus are Gram-positive cocci that comprise of over 50 species and subspecies, some of which are common commensals of various body sites of different animals (Weese, 2013). Although many *Staphylococcus* spp, are of no clinical significance, some are important opportunistic pathogens (Weese, 2013; Busscher et al., 2006). In equine medicine *S. aureus, S. intermedius* and *S. hyicus,* have been associated with clinical infections (Weese et al., 2006). Coagulase-negative species (CoNS) are the predominant commensal staphylococci found in horses, with several species colonising their mucous membranes. Although coagulase-positive staphylococci (CoPS) are the most important groups associated with severe infections with a lower prevalence for *S. aureus* and *S. pseudintermedius* only very rarely reported in horses (Oggutu et al., 2017; De Martino et al., 2010). Resistance to a variety of antimicrobial agents is common within the genus. However, resistance to the narrow spectrum b-lactam methicillin is considered of particular significance as it generally signifies resistance to all b-lactams drugs (Maddox et al., 2015). Moreover, all *Staphylococcus* spp., regardless of their coagulase activity, have potential to develop resistance to different classes of antimicrobials used for human and animal treatment. Just like CoPS, resistance to antimicrobials such as gentamycin, macrolides, tetracycline, streptomycin, trimethoprim, sulfamethoxazole and fluoroquinolones is commonly observed among CoNS isolates from pets and horses (Oggutu et al., 2017). Excessive use of broad spectrum antimicrobials has been hypothesized as the main driver of antimicrobial drug resistance in *Staphylococcus* spp. For example, Bagcigil et al. (2007) reported high levels of resistance to multiple antimicrobial agents including β-lactams in horses with previous history of treatment with β-lactams. Failure to complete the course of antimicrobial treatment has also been identified as a risk factor for development of resistance among staphylococcal isolates from horses (Oggutu et al., 2017).

From a public health point of view, there are increasing reports of highly virulent Staphylococcal infections that can be transmitted between horses and humans (Weese et al., 2006). Infections with antimicrobial drug resistant *Staphylococcus* spp. in both equine and human medicine has been associated with high morbidity, mortality and treatment costs. In animals, infections with antimicrobial drug resistant *Staphylococcus* spp. has also been associated with significant animal welfare

implications due to animals staying sick for long periods in event of treatment failures (Oggutu et al., 2017). According to the World Health Organization, the frequency of resistance to first-line drugs that have traditionally been used to treat infections caused by *Staphylococcus* has increased globally (WHO: Antimicrobial Resistrance Fact Sheet. Geneva: Who Health Organizarion). Unfortunately, this resistance is not limited to human medicine, but is being seen more frequently in domestic species, and in equine medicine in particular (Bryan et al., 2010).

Escherichia coli

Escherichia coli, a Gram-negative bacteria normally residing in the intestinal tract, is among the most common pathogenic agents in humans and animals. It is classified into various pathotypes, causing intestinal and extra-intestinal infections, including gastroenteritis, urinary tract infections, skin and soft tissue infections, and septicaemia (Hammerum and Heuer, 2009). Pathogenic *E. coli* can also be found in fecal samples of diarrheic foals suggesting the implication of the microorganism in foal's diarrhea. Furthermore, horses can act as a reservoir of antimicrobial-resistant *E. coli* and its genetic determinants that subsequently pose a risk to human public health through hindering the ability to treat infections (Gharaibeh et al., 2020).

E. coli is intrinsically susceptible to almost all clinically relevant antimicrobial agents, but this bacterial species has a great capacity to accumulate resistance genes, mostly through horizontal gene transfer (Poirel et al., 2018). The most problematic mechanisms in *E. coli* correspond to the acquisition of genes coding for extended-spectrum β-lactamases (conferring resistance to broad-spectrum cephalosporins), carbapenemases (conferring resistance to carbapenems), 16S rRNA methylases (conferring pan-resistance to aminoglycosides), plasmid-mediated quinolone resistance (PMQR) genes (conferring resistance to (fluoro)quinolones), and *mcr* genes (conferring resistance to polymyxins). Infections are usually less responsive to treatment when multidrug-resistant (MDR) *E. coli* are encountered, especially when they are resistant to critically important antimicrobials (Poirel et al., 2018; Saputura et al., 2017).

Enterococci

Enterococci are Gram-positive members of the commensal flora of the gastrointestinal tract of many mammals, including man and horses. However, they are also capable of causing disease and represent a serious cause of nosocomial infections in people, with the most significant species being *Enterococcus faecalis* and *Enterococcus faecium*. All enterococci are intrinsically resistant to

cephalosprins and aminoglycosides, limiting treatment options, which is complicated further by the increasing prevalence of transferable resistance to other antimicrobials (most critically to vancomycin) in some species. Most vancomycin resistant enterococci (VRE) are *E. faecalis* and *E. faecium*, but resistance is occasionally seen in other members of the genus such as *Enterococcus gallinarum*, *Enterococcus durans* and *Enterococcus casseliflavus* (Maddox et al., 2015).

Salmonella

Salmonella enterica is an important cause of disease in adult horses and foals. Clinical signs of salmonellosis in horses include diarrhea, fever, colic, dehydration, and manifestations of septicemia. Horses with peracute salmonellosis can die rapidly despite aggressive therapy. However, many *Salmonella* infections in horses remain subclinical or cause only mild signs of disease (Jones, 2009). Antimicrobials typically used alone or in combination for the treatment of salmonellosis in horses include ceftiofur, enrofloxacin, and gentamicin. However, multidrug-resistant *Salmonella* isolates from horses have been frequently reported, particularly among those serovars that are most commonly associated with clinical disease. Antimicrobial-resistant *Salmonella* isolates limit treatment options and increase the risk for therapeutic failure in veterinary clinical settings (Schott et al., 2001).

Antimicrobial-resistant *Salmonella* isolates obtained from horses also represent a potential threat to public health. Zoonotic transmission of *Salmonella* can occur by direct contact with the feces of infected horses. Transmission through foodborne exposure is also possible (Jones, 2009).

Pseudomonas

Pseudomonas spp. are Gram-negative bacteria comprising more than 200 species, that can be ubiquitously found in humans, animals, soil, and plants. Only a few species are of clinical interest in either humans or animals, and *P. aeruginosa* is by far the most frequently reported pathogen (Lupo et al., 2018). *P. aeruginosa* sporadically causing skin or respiratory infections in horses. It is more frequently associated with genital tract infections such as endometritis, which can lead to reduced fertility or even sterility. Horse-to-horse transmission is a potential source of transmission since nonpathogenic isolates may be incidentally introduced into the vagina of the mare during coitus (Metcalf, 2001).

Multidrug resistance is commonly encountered in *Pseudomonas* species, partly due to widespread intrinsic resistance to agents such as β-lactams and also the high prevalence of multidrug efflux pumps observed. Extensive multidrug resistance has

been seen in *Pseudomonas* isolates recovered from foals with sepsis, with resistance to most of the antimicrobials tested and only amikacin, ticarcillin/clavulanic acid and imipenem remaining active against the majority of isolates (Lupo et al., 2018; Maddox et al., 2015). With carbapenems such as imipenem considered critically important antimicrobials and largely reserved for human medicine, treatment options can be limited for horses (Maddox et al., 2015).

Acinetobacter

The *Acinetobacter* genus includes 50 species of nonmotile Gram-negative rods that are strictly aerobic (Lupo et al., 2018). One species in particular, *Acinetobacter baumannii*, frequently exhibits high-level resistance to a wide range of antimicrobials including aminoglycosides, cephalosporins, fluroquinolone and tetracyclines, with less resistance seen in other members of the genus. Multidrug-resistant isolates of *A. baumannii* have been sporadically reported as causes of wound infections and bronchopneumonia, with sensitivity restricted to trimethoprim-sulfonamide and marbofloxacin in some cases (Lupo et al., 2018; Maddox et al., 2015).

Klebsiella

Klebsiella pneumoniae is a Gram-negative, facultative anaerobic bacterium, which can be found in the normal flora of the mouth, skin, and intestines, but also, it can cause destructive changes to human and animal lungs (Vo et al., 2007).

Among MDR strains, extended spectrum ß-lactamase (ESBL) producing *Klebsiella pneumoniae* are frequently implicated in nosocomial infections thus, not surprisingly, being considered by the World Health Organization as a major global concern (WHO: World Health Organisation). *K. pneumoniae* is considered a commensal agent in horses and its clinical relevance and disease severity depend on the strain's pathogenic potential. Additionally, horses have been shown to carry human associated MDR *K. pneumoniae* and ESBL-producing bacteria, but characterization of MDR isolates are still lacking for this particular species, with only a few reports of ESBL-producing *K. pneumoniae* in horses available to date. In research by Trigo de Roza et al. the *K. pneumoniae* isolate, obtained from the surgical site infection, was resistant to the majority of the antimicrobials tested, which included different cephalosporins, fluoroquinolones and aminoglycosides (Trigo de Roza et al., 2019).

Conclusion

AMR is a global problem with implications for both human and equine health. High levels of resistance in equine pathogens and complicating therapy with commonly used antimicrobial agents are big challenge. This underlines the importance of individual bacteriological and antimicrobial susceptibility testing as well as minimizing and optimizing the antimicrobial therapy in horses to reduce further development of resistance.

Because microorganisms may serve as a reservoir for AMR in all ecological niches, a "one health" coordinated multisectorial approach is desired to investigate and address this warning phenomenon. This approach appears to be a winning strategy to combat and reduce the burden of AMR, but it requires combined forces and resources that are consistently and effectively implemented by both human and veterinary health professionals.

References

1. Bagcigil FA, Moodley A, Baptiste KE, Jensen VF, Guardabassi L. 2007. Occurrence, species distribution, antimicrobial resistance and clonality of methicillin- and erythromycin-resistant staphylococci in the nasal cavity of domestic animals. Vet Microbiol;121:307–15. 2. Busscher JF. Van Duijkeren E, Sloet van Oldruitenborgh-Oosterbaan MM. 2006. The prevalence of methicillin-resistant staphylococci in healthy horses in the Netherlands. Vet. Microbiol. 113:131–6. 3. Bowen M. 2013. Antimicrobial stewardship: time for change Equine Vet. J., 45, pp. 127-129 4. Bryan J, Leonard N, Fanning S, Katz L, Duggan V. 2010. Antimicrobial resistance in commensal faecal Escherichia coli of hospitalised horses. Ir Vet J.;63(6):373–9. 5. Chipangura J.K. , H. Eagar, M. Kgoete, D. Abernethy, V. Naidoo. 2017. An investigation of antimicrobial usage patterns by small animal veterinarians in South Africa Prev. Vet. Med., 136, pp. 29-38. 6. De Martino, L., Lucido, M., Mallardo, K., Facello, B., Mallardo, M.,Iovane, G., Pagnini, U., Tufano, M.A. and Catalanotti, P. 2010. Methicillin-resistant staphylococci isolated from healthy horses andhorse personnel in Italy.J. Vet. Diagn. Invest.22, 77-82. 7. Gharaibeh, M. H., Abutarbush, S. M., Mustafa, F. G., Lafi, S. Q., & Halaiqa, M. S. 2020. Identification of risk factors associated with antimicrobial resistance in equine fecal Escherichia coli isolates. Infection, Genetics and Evolution, 104317. doi:10.1016/j.meegid.2020.104317 8. Hammerum A. M., O.E. Heuer. 2009. Human health hazards from antimicrobial-resistant Escherichia coli of animal origin Clin. Infect. Dis., 48, pp. 916-921 9. Hughes L.A. , G. Pinchbeck, R. Callaby, S. Dawson, P. Clegg, N. 2013. Williams Antimicrobial prescribing practice in UK equine veterinary practice Equine Vet. J., 45, pp. 141-147 10. Jones SL. 2009. Acute diarrhea. In: Smith BP ed. Large animal internal medicine. 4th ed. St. Louis: Mosby Elsevier; 743–744. 11. Johns and Adams, 2015 I.C. Johns, E.L. Adams Trends in antimicrobial resistance in equine bacterial isolates: 1999–2012 Vet. Rec., 176 (13) , p. 334 12. Lupo, Agnese; Haenni, Marisa; Madec, Jean-Yves. 2018. Antimicrobial Resistance in Acinetobacter spp. and Pseudomonas spp.. Microbiology Spectrum, 6(3), –. doi:10.1128/microbiolspec.ARBA-0007-2017 13. Metcalf ES. 2001. The role of international transport of equine semen on disease transmission. Anim Reprod Sci 68:229–237 http://dx.doi.org /10.1016/S0378-4320(01)00159-2. 14. Maddox, T. W., Clegg, P. D., Williams, N. J., & Pinchbeck, G. L. 2015. Antimicrobial resistance in bacteria from horses: Epidemiology of antimicrobial resistance. Equine Veterinary Journal, 47(6), 756–765. doi:10.1111/evj.12471 15. Oguttu, J. W., Qekwana, D. N., & Odoi, A. 2017. An Exploratory Descriptive Study of Antimicrobial Resistance Patterns of Staphylococcus Spp. Isolated from Horses Presented at a Veterinary Teaching Hospital. BMC Veterinary Research, 13(1). doi:10.1186/s12917-017-1196-z 16. Poirel, L., Madec, J.-Y., Lupo, A., Schink, A.-K., Kieffer, N., Nordmann, P., & Schwarz, S. 2018. Antimicrobial Resistance in Escherichia coli . Microbiology Spectrum, 6(4). doi:10.1128/microbiolspec.arba-0026-2017 17. Saputra, S., Jordan, D., Mitchell, T., Wong, H. S., Abraham, R. J., Kidsley, A., … Abraham, S. 2017. Antimicrobial resistance in clinical Escherichia coli isolated from companion animals in Australia. Veterinary Microbiology, 211, 43–50. doi:10.1016/j.vetmic.2017.09.014 18. Schott HC II, Ewart SL, Walker RD, et al. 2001. An outbreak of salmonellosis among horses at a veterinary teaching hospital. J Am Vet Med Assoc; 218: 1152–1159. 19. Steinman, A., & Navon-Venezia, S. (2020). Antimicrobial Resistance in Horses. Animals, 10(7), 1161. doi:10.3390/ani10071161 20. Toombs-Ruane L. J., C.B. Riley, A.T. Kendall, C.F. Bolwell, J. Benschop, S.M. Rosanowski. 2015. Antimicrobial susceptibilities of aerobic isolates from respiratory samples of young New Zealand horses J. Vet. Intern. Med., 29 (6), pp. 1700-1706 21. Vincze, S., Stamm, I., Kopp, P.A., Hermes, J. and Adlhoch, C. (2014)Alarming proportions of methicillin-resistantStaphylococcus aureus(MRSA) in wound samples from companion animals, Germany 2010-2012.PLoS One9, e85656 22.Vo, A. T. T., van Duijkeren, E., Fluit, A. C., & Gaastra, W. 2007. Characteristics of extended-spectrum cephalosporin-resistant Escherichia coli and Klebsiella pneumoniae isolates from horses. Veterinary Microbiology, 124(3-4), 248–255. doi:10.1016/j.vetmic.2007.04.027 23. Weese JS. 2013. Staphylococcal infections. In: Sellon DC, Long M, editors. Equine Infect Dis Internet. Toronto: Elsevier Health Sciences; . p. 664. 24. Weese JS, Caldwell F, Willey BM, Kreiswirth BN, McGeer A, Rousseau J, et al. 2006. An outbreak of methicillin-resistant Staphylococcus Aureus skin infections resulting from horse to human transmission in a veterinary hospital. Vet Microbiol.;114:160–4. 25. WHO: Antimicrobial Resistrance Fact Sheet. Geneva: Who Health Organizarion.

Prirodom za negu životinja

NUTRITION FEIX FEED ADDITIVES

- ŽIVE ĆELIJE KVASCA
- ORGANSKI SELEN
- VEZIVAČ MIKOTOKSINA
- MOS

ActiBeet®

A TRADEMARK OF AGRANA



Phileo by Lesaffre

ActiSaf Sc 47

www.feix.rs

AEH
Cleanactiv
Phito csc
Pulmocare
Program za živinu

Ruminolac
Pulmofit
Pulmo cx
Program za preživare

Colifit
Pulmofit
Program za svinje

Program za ribe

- Pritisak parazita
- Digestivni poremećaj
- Pitanja valorizacije hrane
- Pitanja reprodukcije
- Izazovi laktacije
- Respiratorni poremećaji
- Zootehnički izazovi
- Stres

www.feix.rs