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Article

Antimicrobial resistance of *Staphylococcus* and *Enterococcus* Bacteria in Rural Dogs in Hungary—A Preliminary Report

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Abstract: Antimicrobial resistance (AMR) is one of the most relevant health challenges globally. Since resistant bacteria and their resistance genes circulate through the ecosystem, AMR is among the main focuses of One Health. Dogs are the best friends of humans, therefore their relationships with the owners are mostly very close. This connection can make the dogs vehicles of AMR between the environment and humans. Based on this hypothesis, we investigated faecal samples from 37 dogs in Inner Somogy, Hungary. We isolated and investigated for antibiotic susceptibility 21 and 6 strains of *Staphylococcus* and *Enterococcus* genera, respectively. Among staphylococci and enterococci, 12 and 3 strains proved to be resistant to at least one antibiotic. Multidrug resistant strains were detected only among coagulase negative staphylococci, mainly in *S. sciuri* species. The antibiotics that proved to be inefficient against the most strains were benzylpenicillin (8 strains), moxifloxacin (6 strains), clindamycin (5 *S. sciuri* strains), and fusidic acid (12 strains). In the case of moxifloxacin and fusidic acid, the MIC excessed the EUCAST clinical breakpoint. Analysing the epidemiological background of the animals, outdoors keeping and higher income level of the owners seemed risk factors of AMR carrying, though the sample size of this study could not confirm statistically the apparent interdependence.

Keywords: *Staphylococcus sciuri*; antimicrobial resistance; moxifloxacin; fusidic acid

1. Introduction

Antimicrobial resistance (AMR) is a major global health challenge, which needs a holistic approach, whereas resistant bacteria and resistance genes can spread among the domains of health unstoppably. Therefore, AMR is one of the main focuses of One Health research (ABDULLAHI ET AL., 2021). *Staphylococcus* genus as a model group of bacteria in One Health research is confirmed (CUNY ET AL., 2024), while enterococci proved to be promising as indicators of bacterial (SCHWARTZMAN ET AL., 2023) and resistance circulation (ZAIDI ET AL., 2024) between the elements of the ecosystem.

The total population of dogs worldwide is cca. 700 million individuals and their role as carriers of staphylococci is a well-known phenomenon (AKARSU ET AL., 2024). The transmission of AMR bacteria between dogs and their owners has been studied, yet (SILVA ET AL., 2022), while dogs as potential reservoirs of AMR staphylococci (ELNAGEH ET AL., 2020) and enterococci (DAMBORG ET AL., 2008) were also confirmed.

The bacteria of both genera, *Staphylococcus* and *Enterococcus*, are ubiquitous, halotolerant and less fastidious during culture (CHOI ET AL., 2014; ZAIDI ET AL., 2024). Accordingly, both groups can serve as a good model in One Health AMR investigations (CUNY ET AL., 2024; ZAHEER ET AL., 2020).

Our hypothesis was that rural dogs can perform a vehicle function for AMR between the natural environment and the owners. In this pilot study, we collected faecal samples from village dogs to survey the prevalence of AMR staphylococci and enterococci in this host population. Additionally, this research aimed to reveal potential risk factors in the background of AMR carrying.

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2. Materials and Methods

Faecal samples were collected from outdoor kept rural dogs in a small village, Ötvöskónyi in Inner Somogy, Hungary. The village is located in the South Transdanubia, one of the less developed regions of Hungary (CSIZMADIA & BAREITH, 2021; MOLNÁR & HAJDÚ, 2024). The pattern of sampling was designed to represent the varied culture of care characteristic for different types of households of the village. Thus, we collected samples from both the central zones and a segregated section at the periphery of the settlement. This section of the village is inhabited by a minority ethnic group mostly in poverty.

All dog owners were informed about the aims of the investigation and asked for permission to take a sample. All dogs were apparently healthy and were not under antibacterial treatment. Parallelly to sampling, we carried out a short questionnaire survey, by which we collected information about the income level of the owner, the age of the dog, the circumstances of keeping (outdoor or indoor), and feeding (controlled or free-choice). Our hypothesis was that those dogs, which live outdoors, especially if the owner leaves the daily portion in front of the animal, could acquire bacterial infection from environmental sources, such as birds or rodents that visit dog food.

The faecal samples were collected from the environment of the dogs and put into plastic bags and transferred into the laboratory in two hours after acquisition. In the laboratory, a sterile cotton swab was plunged into the faecal material and put into a sterile tube filled with Buffered Peptone Water (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) supplemented with 10% sodium chloride. The samples were incubated at 36 °C for 24 hours. After this enrichment step, we homogenised the broth media and by a cotton swab the inoculum was placed on the surface of Plate Count Agar (PCA) solid medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) by streak plate method (SANDERS, 2012).

The inoculated media were incubated for 48 hours at 36 °C. We selected suspect *Staphylococcus* and *Enterococcus* colonies for transfer to a next PCA medium for purification. In the case of staphylococci, 1-3 mm round, convex, opaque, colonies with smooth surface and entire edge were chosen for transfer (SHAW, 1951; AGARWAL ET AL., 2022). In the case of enterococci, small, smooth, round, creamy or translucent colonies were selected (SWAN, 1954; COLLINS ET AL., 1984; RAHMAN ET AL., 2017). After 24 hours of incubation at 36 °C, we evaluated pureness of the isolates. From pure cultures, we performed Gram staining. All bacterial isolates, which proved to be Gram positive cocci were processed by VITEK Compact 2 identification. For this procedure, we used a GP identification card type.

Those isolates, which were determined to belong to the genera *Staphylococcus* or *Enterococcus* with a probability at least 90%, underwent an antimicrobial susceptibility testing. For this step, we applied AST-P592 card type of VITEK Compact 2 system.

The bacteriology results were gathered in an Excel (Microsoft Excel version 2410) file. Based on the data, we determined:

- apparent prevalence of staphylococci and enterococci in the investigated dog population and its subpopulations (central and peripheral zone)
- similarity of bacterial species composition between the subpopulations of the centrally and peripherally kept dogs by calculation of Sorensen-Dice Index, $SDI = 2\times|X\cap Y| \div (|X|+|Y|)$, where $|X\cap Y|$ means the number of those bacteria, which are common between the two subpopulations, while $|X|$ and $|Y|$ mean the numbers of bacteria in the two groups separately (DIAS ET AL., 2021).
- AMR in different bacterial species
- the number of multidrug resistant isolates as the number of those isolates that were resistant to at least three classes of tested antibiotics.

The difference of antimicrobial communities between subpopulations of the investigated dog population was presented in Sankey charts of the MS Excel Power User. The true prevalence intervals

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were calculated by Sterne's exact method (LANG & REICZIGEL, 2014; REICZIGEL, 2003). The difference between the population sections in prevalence of staphylococci, enterococci, AMR, MDR was calculated with Fisher exact test in R-statistics (R for Windows 4.4.2).

3. Results and Discussion

Based on the hypothesis that pet animals can be reservoirs and transmitters of resistant bacteria between different domains of health, we collected faecal samples from 37 rural dogs, 13 and 24 samples from the peripheral and the central section of the settlement, respectively. We could isolate halophilic bacteria from each sample. In four samples, we found other than Gram positive cocci. Four samples contained Gram positive cocci, which could not be identified by the VITEK 2 Compact system. In two samples, *Aerococcus viridans* and *Rothia kristinae* could be detected. In 28 samples (11 peripheral and 17 central), we found bacteria that belonged to the genera *Staphylococcus* (N=22, 59.46%, CI95: 43.19-74.61%) or *Enterococcus* (N=7, 18.92%, CI95: 9.06-34.96%). In five dogs (three peripheral and two central), we detected two strains that belonged to either staphylococci or enterococci.

Coagulase positive staphylococci (CoPS) (*S. aureus* and *S. pseudintermedius*) were isolated from only 2 dogs. Both proved to be sensitive to all tested antibiotics. These findings differ from previous ones. Even in healthy dogs, *S. aureus* is detected very frequently with 19-28% of prevalence and high (50%) rate of methicillin resistance (ELNAGEH ET AL., 2020; SILVA ET AL., 2022; AKARSU ET AL., 2024). The occurrence of other CoPSs is also common in dogs, e. g. *Staphylococcus pseudintermedius* is detected frequently (AKARSU ET AL., 2024; SILVA ET AL., 2022), often with phenotypic methicillin resistance (SILVA ET AL., 2022). In our study, only two CoPS species, *S. aureus* and *S. pseudintermedius*, were detected without any phenotypic AMR to the tested antibiotics.

The coagulase negative staphylococci (CoNS) were represented by the following species: *S. cohnii* ssp. *cohnii*, *S. equorum*, *S. lentus*, *S. sciuri*, *S. simulans*, *S. vitulinus*, and *S. xylosus*. In *S. simulans* and *S. vitulinus* strains, we did not detect resistance to the tested antibiotics. Among the strains of the other species, 12 isolates showed resistance to at least one antibiotic. Moreover, in *S. cohnii* ssp. *cohnii*, *S. equorum*, and *S. sciuri*, we confirmed multidrug resistance in 7 isolates. Among the five *S. sciuri* strains, all were MDR and three of them were resistant to four tested antibiotics. The clindamycin resistance of these *S. sciuri* strains varied between ≤0.25 and 0.5 mg/L minimal inhibitory concentration (MIC). This finding agrees with the well-known phenomenon that *S. sciuri* group, which contains the species *S. sciuri*, *S. lentus*, *S. vitulinus*, *S. fleuretti*, and *S. stepanovicii*, plays as a reservoir of more resistance genes (BECKER ET AL., 2014; ELNAGEH ET AL., 2020). These bacterial species are among the most primitive taxa of the *Staphylococcus* genus. It is hypothesised that most of the resistance genes characteristic for the *Staphylococcus* genus evolved in these species (NEMEGHAIRE ET AL, 2014). Despite the presence of resistance genes, e.g. *mecA*, these species rarely show phenotypic resistance (BECKER ET AL., 2014).

Our observations partly agree with these works, whereas in the case of clindamycin and benzylpenicillin, the detected MICs were 0.25-0.5 and 0.06-0.12 respectively, which did not reach the EUCAST clinical breakpoints (EUCAST, 2024). This might be the result of intrinsic chromosomal resistance with partial expression in phenotypic features (NEMEGHAIRE ET AL, 2014). On the other hand, MIC of moxifloxacin and fusidic acid phenotypic resistance proved to be above the clinical breakpoints suggesting that these are acquired attributes of the isolated strains. This hypothesis is supported by the fact that this resistance quality also occurred in strains of *S. cohni* ssp. *cohni*, *S. equorum*, and *S. lentus* species.

The antibiotics, which proved to be inefficient against the most isolates were benzylpenicillin (8 strains), moxifloxacin (6 strains), clindamycin (5 *S. sciuri* strains), and fusidic acid (12 strains). Moxifloxacin and fusidic acid resistance could be detected in *S. cohnii* ssp. *cohnii*, *S. lentus*, and *S. sciuri* strains with MIC above the EUCAST clinical breakpoints, 0.25 mg/L for moxifloxacin and 1 mg/L for fusidic acid (EUCAST, 2024). Whereas fusidic acid is not frequently applied as an antimicrobial, the unusually high prevalence of fusidic acid resistance in CoNS (57.14%, CI95: 35.44-76.73%) needs further investigations.

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For widespread moxifloxacin resistance among the AST-tested isolates (28.57%, CI95: 13.25-50.60), the close vicinity (~5 km) of a large-scale industrial pig farm might be responsible. The use of fluoroquinolones, especially enrofloxacin, is ubiquitous in the swine industry (LEI ET AL., 2017). Fluoroquinolone resistance of staphylococci was investigated mostly in small animal practice. These studies confirmed that cross-resistance to different fluoroquinolone molecules exists (YOO ET AL., 2010; KANG ET AL., 2014). However, most observations suggest that the rate of resistance against enrofloxacin is always higher in bacterial communities than moxifloxacin resistance (KANG ET AL., 2014; AZZARITI ET AL., 2022). This phenomenon indicates a slower development of resistance to moxifloxacin than to enrofloxacin. In this study, we could not investigate enrofloxacin because the used card type did not contain this antimicrobial. However, the experiences that are gathered by previous works give the impression that resistance to veterinary used fluoroquinolones (enrofloxacin) should be a more severe problem within the study site.

The source or the vehicle of fluoroquinolone resistance spread could not be determined by this research. Those dogs, which carried moxifloxacin resistant staphylococci were all kept outdoors, though one of them was fed in a controlled manner. We deemed controlled feeding as a risk mitigating factor in AMR transmission, since it could impede the contamination of food by vector species. However, other transmitters, e.g. dust or surface water, could not be excluded in outdoor keeping of animals.

Enterococci were isolated from only six dogs. This observation differs from previous studies, whereas enterococci, especially *E. faecalis* are common commensals of animals (NOCERA ET AL., 2022; ZAIDI ET AL., 2024). The exceptionally low number of isolated enterococci could be explained by the pre-enrichment step of our bacteriological protocol. We hypothesised that salt tolerance of these bacteria (NOCERA ET AL., 2022; PARIA ET AL., 2022) allows the application of 10% salt in buffered peptone water to avoid growth of concurrent bacteria. In future research, a salt tolerance test should be carried out to determine the concentration of salt during pre-enrichment, which can mitigate the growth of contaminating flora without growth reduction of enterococci.

The found species were *E. casseliflavus*, *E. faecalis*, *E. faecium*, *E. villorum*. Both *E. casseliflavus* strains that could be isolated were vancomycin resistant with low (≤0.25 mg/L) MIC, which is not considered a unique attribute of this species, whereas it contains *vanC* gene group on its chromosome, which confers resistance to vancomycin (SOARES DE MEDEIROS LIMA ET AL., 2024). The very low MIC of our isolates also supported the hypothesis that the detected resistance is an intrinsic and not an acquired feature. The only *E. faecalis* strain proved to be gentamicin resistant, which is possibly an acquired characteristic. Although without molecular genetic investigation, this cannot be claimed. The other three strains showed susceptibility to all tested antibiotics. The details of isolated species and antimicrobial resistance were summarised in Supplementary material, which can be accessed at link https://zenodo.org/records/14212483.

Based on the questionnaire survey, we ascertained that the age of the dog did not show any correlation with the isolated bacterial species or the antimicrobial resistance features of the bacteria, whereas all species, all resistance phenotype, the infection with multiple strains, and bacteria with multiple antimicrobial resistance could be detected in all age classes uniformly.

All four dogs that can spend time in the owner's house were fed in controlled circumstances, mostly indoor. Among outdoors kept animals, four were fed in a controlled manner, by which the access of potential vector species (birds or rodents) to their feed could be excluded. Those dogs, which were fed under the owner's check, carried 2 AMR strains, both MDR. Among the other 29 dogs, which were fed outdoors in a free-choice manner, 13 carried AMR strains, 5 of them with MDR phenotype. Fisher's exact test comparison of the groups fed in a controlled manner and free-choice regime could not reveal difference (P=0.43).

The socioeconomic environment of the dogs was analysed in two ways. We compared them by localisation within the settlement (central or peripheral) and by their owner's income (below the minimum wage or higher). Comparing the *Enterococcus-Staphylococcus* communities of different localities, we found that 12 and 5 species could be identified from the central and peripheral regions, respectively, while 5 species were shared between the two localities. Therefore, the Sorensen-Dice

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index for the similarity of localities proved to be 0.53 indicating a moderate overlap. Comparing dog populations that were kept by low- and higher-income households, 6 and 11 bacterial species could be identified, respectively, with 3 species shared between the two groups. Thus, the SDI proved to be 0.35 indicating some difference between the bacterial communities of the two subpopulations. The Sankey charts representing the different bacterial communities of the compared dog subpopulations can be accessed at link https://zenodo.org/records/14212483.

Regarding antimicrobial resistance, we could find the same pattern when we compared either the centre and periphery or low- and higher-income owners. Among the 37 investigated animals, 13 belonged to low-income owners and also 13 lived on the peripheries of the settlement, though not the same 13. Among the dogs of poor owners, 4 carried AMR bacteria of which 2 were MDR. The situation was the same for dogs of the peripheries (4 AMR of which 2 MDR). Among the dogs of higher income owners and those who lived in the central zones of the settlements, 10 carried AMR bacteria, of which 5 proved MDR. Statistical analysis with Fisher's exact test provided P=0.73. Therefore, the apparently more disadvantageous AMR situation in the central zones and in higher income households was not confirmed by these data.

4. Conclusions

In this study, we investigated faecal materials of rural dogs to analyse the bacterial communities belonging to the genera *Staphylococcus* and *Enterococcus*. The main limitation of this study was the small sample size. Though we could notice some interesting apparent phenomena in the epidemiological background of AMR, we could not confirm or exclude those. Thus, the effect of socioeconomic circumstances, keeping and feeding regime on AMR cannot be analysed appropriately with this number of samples. Another particularly important shortcoming originated from the sample preparation process, especially the pre-enrichment step in buffered peptone water supplemented with 10% sodium chloride. This protocol might have caused small numbers of isolated *Enterococcus* and CoPS strains. For elimination of this bias, exact salt tolerance of faecal originated *Staphylococcus* and *Enterococcus* strains must be determined before continuing this study.

However, as a pilot study, it could confirm that bacteria belonging to the genera *Staphylococcus* and *Enterococcus* can be good indicators of AMR in an animal population. By molecular genetic analysis of strains and geospatial analysis of localisation of the isolates' origin, researchers can gain appropriate information on AMR transmission in the ecosystem.

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