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RESEARCH REGARDING THE RESISTANCE PHENOTYPES OF BACTERIA ISOLATED FROM DOGS WITH RESPIRATORY TRACT INFECTIONS

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Abstract

The resistance phenotypes to animal pathogenic bacteria (both Gram-positive and Gram-negative bacteria) are increasing in frequency due to the use of antibiotic-based veterinary medicinal products in both in farm animals and pets. The research aimed to establish phenotypically the antibiotic resistance in bacterial strains isolated from dogs with various respiratory tract infections. Both susceptible Gram-positive and Gram-negative isolated strains had the highest frequency to enrofloxacin (82.45% and 81.81%). Gram-positive resistant strains had the highest frequency to penicillin G (70.18%), while Gram-negative strains had the highest frequency of resistance to lincomycin. The results confirm the marked increase of resistance phenotypes in both Gram-positive and Gram-negative strains to a wide range of antimicrobial substances, frequently used in the therapy of infectious diseases in dogs.

Key words: bacteria, dogs, resistance phenotypes, respiratory tract

Antimicrobial resistance in bacteria represents a very topical problem in veterinary and human medicine, since is considered a phenomenon with pronounced zoonotic risk. The resistance phenotypes to animal pathogenic bacteria (both Gram-positive and Gram-negative bacteria) are increasing in frequency due to the use of antibiotic-based veterinary medicinal products in both in farm animals and pets (Aarestrup, F., M., 2006; Guardabassi, L., Courvalin, P., 2006; Markey, B., *et al*, 2013; Riedel, S., *et al*, 2019; Schwarz, S., *et al*, 2006, Viţălaru, A., B., 2020).

The expansion of multiple antibiotic resistance, in bacterial species pathogenic for animals and humans, led to extensive phenotypic and genotypic studies to clarify, as deeply this phenomenon. Thus, it was demonstrated that antibiotic resistance is genetically encoded, supported by many resistance genes present in the bacterial chromosome and in mobile genetic elements (plasmid R, intergons, transposons). Through it, the genes can be transferred between strains of the same bacterial species (intraspecific transmission), as well as between strains belonging to other bacterial species, respectively interspecific transmission (Aarestrup F., M., 2006; Arber, W., 2014; Guardabassi, L., Courvalin, P., 2006; Schwarz, S., *et al*, 2006).

In recent years, the antibiotic resistance of a large number of bacterial germs has become a global threat to public health. Among the bacteria that represent the greatest threat to human health, due to the increase in antibiotic resistance, there are several Gram-positive bacteria, such as *Staphylococcaceae* or *Streptococcaceae* family, as well as Gram-negative bacteria, included in *Enterobacteriaceae* family, especially *Escherichia coli*, *Salmonella* spp. or *Klebsiella* spp. (Cummings, K., *et al*, 2015; Guardabassi, L., Courvalin, P., 2006; Li, Y., *et al*, 2021; Schwarz, S., *et al*, 2006; Mavrides, D. E., *et al*, 2021).

The research aimed to establish phenotypically the antibiotic resistance in bacterial strains isolated from dogs with various respiratory tract infections.

MATERIAL AND METHOD

The samples with pathological material were obtained from dogs with various diseases of the respiratory tract (cough, tachypnea, dyspnea or various secretions), and the sampling was carried out before starting the therapy with antimicrobial substances, or in case it was started, 48 hours after its interruption. Therefore, a total of 70 samples were collected from dogs.

For the isolation of primary cultures, samples with pathological material were inoculated in nutrient broth and incubated at a temperature of 37°C, under aerobic conditions, for 18-20 hours. Next, to identify the bacterial species, inoculations were made on CHROMagar Orientation medium, a

chromogenic, non-selective culture medium used for the direct qualitative detection of some pathogenic bacteria.

After incubation, the plates were examined, respectively the cultural characters of the colonies were observed, and the genera and bacterial species were preliminarily identified. To confirm the isolated colonies on this medium, inoculations were made on other media, such as Chapman, 7% defibrinated sheep blood agar, Levine, acetamide agar and MacConkey.

Gram-stained smears were made from the characteristic isolated colonies, to examine the morphological characters and confirm the genera or species. Subsequently, inoculations were made on 7% defibrinated sheep blood agar to obtain fresh strains in pure cultures, used for the susceptibility testing to antimicrobial substances.

All isolated and identified bacterial strains were tested for susceptibility to the following antimicrobial substances: aminoglycosides - streptomycin (S), kanamycin (K), gentamicin (GM); *β*- lactams - ampicillin (AMP), amoxicillin with clavulanic acid (AMC), penicillin G (P); cephalosporins - cefadroxil (CDX), cephalexin (CN), cefquinome (CFQ); phenicols - chloramphenicol (C); lincosamides - lincomycin (L); macrolides - clindamycin (CD), erythromycin (E); quinolone - enrofloxacin (ENR); sulfonamides - trimethoprim/sulfamethoxazole (SXT); tetracyclines - tetracycline (TE), doxycycline (DO).

These 17 antimicrobial substances, used to determine the resistance profile, were chosen according to: form and mode of administration, therapeutic characteristics and effectiveness, degree of absorption, as well as availability.

The susceptibility testing was done with the disk-diffusimetric method (Kirby-Bauer method) using the Mueller-Hinton medium, respectively biodiscs with the antimicrobial substances mentioned above, kept tightly closed and refrigerated at 4-8˚C. Thus, a total number of 90 isolated strains, included 8 bacterial species, were tested.

Comparisons between the prevalence of antibiotic resistance Gram positive and negative strains were performed using the Chi-square test at a level of significance set at $p<0.05$.

RESULTS AND DISCUSSIONS

From the primary cultures, obtained in nutrient broth, inoculations were made, with the bacteriological loop, by dispersion on the CHROMagar Orientation medium. A total of 90 bacterial strains (from a total of 60 positive samples) and a number of 10 sterile samples were isolated. Thus, the strains could be classified into three Gram positive genera (*Enterococcus*, *Staphylococcus* and *Streptococcus*), respectively into four Gram negative genera (*Escherichia*, *Klebsiella*, *Proteus* and *Pseudomonas*).

To confirm the species, inoculations were carried out on special media mentioned previously, and after smears stained by the Gram method. The results showed germs with shape and characteristic grouping of each species, stained Gram positive, respectively Gram negative.

Thus, based on the bacteriological and bacterioscopic examinations carried out, respectively based on the morphological and cultural characters developed by the inoculated bacteria, 90 bacterial strains were isolated, classified into 8 species (*table 1*).

Table 1

Bacterial species isolated from dogs

Crt. no	Sample	Identified bacterial species	No. of strains	
			No.	%
	Pharyngeal exudate	**Gram positive species**		
1.		*Enterococcus spp.*	20	22.23
2.		*S. aureus*	26	28.89
3.		*Staphylococcus spp.*	8	8.88
4.		*Streptococcus spp.*	3	3.33
		Gram negative species		
5.		*E. coli*	24	26.67
6.		*Klebsiella spp.*	5	5.55
7.		*Proteus spp.*	2	2.22
8.		*P. aeruginosa*	2	2.22
TOTAL			**90**	**100**

According to results, 90 bacterial strains were isolated from the pathological material samples taken from the pharyngeal exudate of dogs. Thus, a total number of 57 Gram positive species were isolated, namely 20 strains of *Enterococcus* spp., 26 strains of *S. aureus*, 8 strains of *Staphylococcus spp.*, 3 strains of *Streptococcus* spp. Gram negative species were isolated in a number of 33 strains, respectively 24 strains of *E.*

coli, 5 strains of *Klebsiella* spp., two strains of *Proteus* spp. and two strains of *P. aeruginosa*.

The results obtained regarding the susceptibility testing to antimicrobial substances were done according to Gram positive and Gram negative bacterial species, but also according to the class of antibiotics.

Thirteen antimicrobial substances for Gram positive strains and fourteen antimicrobial substances for Gram negative strains, from several classes, were used to identify the resistance phenotypes.

In case of isolated Gram positive species, the results obtained revealed that sensitive strains had a frequency between 29.82% for penicillin G and 82.45% in the case of enrofloxacin. The resistant strains had a frequency between 17.55% for enrofloxacin and 70.18% for penicillin G. All the interpretations were made according to EUCAST 2022 recommendations (*table 2*).

Table 2

The results obtained regarding the susceptibility testing of Gram positive strains

Antibiotic	C (µg)	No. of strains (57)			
		S		R	
		No.	%	No.	%
Kanamycin	30	33	57.89	24	42.11
Gentamicin	10	29	50.87	28	49.13
Ampicillin	10	25	43.86	32	56.14
Amoxicillin + clavulanic acid	20-10	24	42.11	33	57.89
Penicillin G	6	17	29.82	40	70.18
Cephalexin	30	34	59.65	23	40.35
Cefadroxil	30	35	61.40	22	38.60
Chloramphenicol	30	34	59.65	23	40.35
Clindamycin	2	21	36.84	36	63.16
Erythromycin	15	30	52.63	27	47.37
Enrofloxacin	5	47	82.45	10	17.55
Tetracycline	30	31	54.38	26	45.62
Doxycycline	30	31	54.38	26	45.62

Legend: C = concentration S = sensitive strains; R = resistant strains

According to the class of antimicrobial substances, for the group of Gram-positive strains, the results were different. Thus, from the **aminoglycosides** group, the antibiotic resistance testing was made for kanamycin and gentamicin. The resistance of the isolated strains had a similar frequency to the two selected antimicrobial substances, respectively 49.13% to gentamicin and 42.11% to kanamycin.

In case of **β-lactams**, for the isolated Gram-positive bacteria were selected the most antibiotics, to which the resistance phenotypes were determined, namely ampicillin, amoxicillin with clavulanic acid and penicillin G, considering that β-lactams are recommended in the treatment of infections caused by both Gram-positive and Gram-negative bacteria.

Therefore, antibiotic resistance had the highest frequency to penicillin G (70.18%), followed by amoxicillin with clavulanic acid (57.89%) and ampicillin 56.14%. Regarding the frequency of susceptible strains, the highest frequency was for the strains susceptible to ampicillin (43.86%), followed by amoxicillin with clavulanic acid (42.11%) and penicillin G (29.82 %).

From the **cephalosporins** group, two antimicrobial substances, namely cephalexin and cefadroxil, were selected for the identification of resistance phenotypes. Following the results, was observed that the frequency of resistant strains was higher to cephalexin (40.35%) than to cefadroxil (38.60%), but lower than that of sensitive strains to both antimicrobial substances.

In case of **phenicols** group, where resistance phenotypes were made only to chloramphenicol, it was observed that the frequency of antibiotic sensitivity was higher than that of antibiotic resistance.

Two antibiotics were selected from the **macrolide** category, namely erythromycin (indicating the inducible resistance to 14-atom macrolides) and clindamycin (indicating the inducible resistance to 16-atom macrolides). Of the total number of Gram positive strains isolated, the resistant strains had a frequency of 63.16% to clindamycin and 47.37% to erythromycin.

The antibiotic resistance to the **quinolone** group, was done only for enrofloxacin Thus, analyzing the results obtained, was observed that the frequency of Gram positive strains sensitive to

enrofloxacin was the highest, respectively 72.09%, which suggests a reduced use of this antimicrobial substance in the therapy of infections in dogs.

The resistance phenotypes to **tetracycline** antibiotics was established for two antimicrobial substances, namely tetracycline and doxycycline. Therefore, the results revealed the same frequency, in the case of the two antimicrobial substances, the frequency of sensitive strains (54.38%) being slightly higher than that of resistant strains (45.62%).

For the isolated Gram negative strains, the resistance phenotyopes were establised for 14 antimicrobial substances, from nine classes, and the frequency of resistant strains was between 18.19% for enrofloxacin and 81.81% for lincomycin (*table 3*).

Table 3

The results obtained regarding the susceptibility testing of Gram negative strains

Antibiotic	C (µg)	No. of strains (33)			
		S		R	
		Nr.	%	Nr.	%
Streptomycin	10	9	27.27	24	72.72
Gentamicin	10	20	60.61	13	39.39
Ampicillin	10	11	33.33	22	66.67
Amoxicillin + clavulanic acid	20-10	14	42.42	19	57.58
Penicillin G	6	9	27.27	24	72.72
Cephalexin	30	23	69.70	10	30.30
Cefquinome	30	25	75.75	8	24.25
Chloramphenicol	30	21	63.63	12	36.37
Lincomycin	15	6	18.18	27	81.82
Clindamycin	2	10	30.30	23	69.70
Erythromycin	15	16	48.48	17	51.52
Enrofloxacin	5	27	81.81	6	18.19
Doxycycline	30	14	42.42	19	57.58
Trimethoprim/ Sulfamethoxazole	30	12	36.36	21	63.64

Legend: C = concentration S = sensitive strains; R = resistant strains

From the group of **aminoglycosides**, the identification of resistance phenotypes was made for streptomycin and gentamicin and the results were the following: the frequency of resistant Gram negative strains was higher for streptomycin (72.72%) compared to gentamicin (39.39%), which suggests greater use of this antimicrobial substance in infections caused by Gram-negative bacteria.

In case of the **β-lactams** group, the same three antimicrobial substances were also selected for testing the Gram-negative strains. Thus, compared to the resistant Gram positive strains, the Gram negative ones had the highest frequency against penicillin G (72.72%), which indicates the wide use of this antimicrobial substance in the therapy of infections in dogs, regardless of the category of bacteria that produced those infections. However, the frequency of resistant Gram negative strains was made to ampicillin (66.67%), respectively to amoxicillin with clavulanic acid (57.58%).

For the isolated Gram negative strains, from the **cephalosporins** group, two antimicrobial substances were selected, namely cephalexin and cefquinome, which had a relatively close frequency of resistant strains (30.30% to cephalexin and 24.25% to cefquinome), but much lower, compared to the frequency of strains sensitive to these two antibiotics.

In the case of the **phenicols** group, resistance phenotypes were made only to chloramphenicol, where the frequency of susceptible strains was higher (63.63%) than that of resistant strains (36.37%).

Also, in the case of the **lincosamide** group, testing the antibiotic resistance was done only to lincomycin, the obtained results indicating the highest frequency of resistant Gram negative strains and, therefore, a low frequency of sensitive strains.

From the **macrolides** category, the same two antibiotics, erythromycin and clindamycin, were selected, also used for the identification of resistance phenotypes of Gram positive strains. However, the Gram-negative strains had a higher frequency of antibiotic resistance to clindamycin (69.70%) compared to erythromycin, where the frequency was 51.52%.

As in the case of Gram positive strains, the sensitive Gram negative strains also had the highest frequency of 81.81% to enrofloxacin, the antimicrobial substance used from the **quinolone** group, while the resistant strains had a frequency of only 18.19%.

The resistance phenotypes for the Gram negative strains, from the **tetracycline** group, were established for doxycycline and had a higher frequency than that of sensitive phenotypes towards this antibiotic.

From the **sulfonamides** group, the trimethoprim with sulfamethoxazole was selected, for the identification of resistance phenotypes, with the following values: a frequency of 63.64% for the resistant Gram negative strains, respectively a frequency of 36.36% in the case of sensitive strains.

Statiscally, was noticed that there is no association ($p>0.05$) between Gram positive strains, respectively Gram negative strains and the behavior towards antibiotics that were common to the two categories of isolated strains: GM X^2 (1, N=90) = 0.79, AMP X^2 (1, N=90) = 0.96, AMC X^2 (1, N=90) = 0.0009, P X^2 (1, N=90) = 0.0003, CN X^2 (1, N=90) = 0.90, C X^2 (1, N=90) = 0.13, CD X^2 (1, N=90) = 0.39, E X^2 (1, N=90) = 0.14, ENR X^2 (1, N=90) = 0.005 and DO X^2 (1, N=90) = 1.19.

Multi-resistant strains have an increasing frequency and the identification of these resistance phenotypes to the antibiotics used in therapy is a very important aspect, as it indicates the continuous expansion of this phenomenon through the two-way animal-human epidemiological circuit. Thus, numerous research teams focus on the identification of multi-resistant strains, both Gram positive and Gram negative, as well as their portage from animal to human and vice versa (Bertelloni, F., *et al,* 2021; Marchetti, L., *et al*, 2021*;* Moon, D.-C., *et al*, 2022; Murray, A., K., *et al,* 2019).

For example, the study by Roca L. et al. aimed to determine the antibiotic resistance in strains of pathogenic bacteria isolated from dogs. Thus, the susceptibility to antibiotics of the isolated strains was determined by the disc-diffusimetric method, on a total number of 81 bacterial strains, the most common species being *Staphylococcus intermedius, Pseudomonas aeruginosa* and *Escherichia coli*. The results indicated the resistance of these species to some antibiotics, respectively: *S. intermedius* was resistant to trimethoprim/sulfamethoxazole (31%) and enrofloxacin (23%), *P. aeruginosa* was resistant to cephalexin (86%) and clindamycin (76 %) and *E.coli* was resistant to clindamycin (78%) and trimethoprim/sulfamethoxazole (75%). Therefore, Gram-negative species demonstrated the highest frequencies of resistance (*Roca, L., et al, 2017).*

A study done by Pedersen K. et al., on samples from dogs, provided data on the emergence of antibiotic resistance in important pathogens. Resistance to cephalosporins and amoxicillin with clavulanic acid was decreased for almost all bacterial species examined, except for *P. aeruginosa*. Of the isolated *S. intermedius* samples, 60.2% were resistant to penicillin, 30.2% to fusidic acid and 27.9% to macrolides and in *E. coli* samples, the highest resistance was reported to ampicillin, sulfonamide, tetracyclines and streptomycin (Pedersen, K., *et al*, 2007).

Daodu O. B. et al. investigated the antibiotic resistance profile of 41 strains of *E. coli* from 173 samples collected from the respiratory tract of clinically healthy dogs. Thus, antibiotic resistance had the following values: amoxicillin with a frequency of 53.7% of resistant strains, chloramphenicol with a frequency of 22%, respectively gentamicin with a frequency of 29.3%. Likewise, on another study, on the antibiotic susceptibility of bacteria isolated from 502 dogs with respiratory symptoms, , Rheinwald, M. et al. identified *E. coli* strains with an antibiotic resistance to enrofloxacin (72.5%), to gentamicin (70%), to cephalexin (50.06%), to amoxicillin/clavulanic acid (39.1%), to trimethoprim/sulfomethoxazole (47.5%), to doxycycline (27.5%), respectively to ampicillin (32.4%) (Daodu, O., B., *et al*, 2016; Rheinwald, M., *et al*, 2014).

In the research carried out by Qekwana D. N. et al., on 157 dogs with lower respiratory tract infections, the authors identified 162 bacterial strains. Almost all isolated strains (99.5%) showed resistance to at least one antibiotic and 64.7% were multi-resistant, with resistance to penicillin G (90.9%), lincomycin (100%), tylosin (75.8%), lincospectin (73.7%), ampicillin (72.5%) and kanamycin (68.4%) (QEKWANA, D. N., *et al*, 2020).

Therefore, the results obtained regarding the resistance phenotypes to Gram positive and Gram negative strains isolated from dogs with different respiratory tract diseases underline the importance of identifying these strains, which may have a zoonotic character. Thus, pets can act as a real microbial reservoir for humans, especially their owners, but also vice versa, from humans to pets, demonstrating this complex epidemiological circuit existing in both Gram-positive and Gram negative bacterial species.

CONCLUSIONS

Both susceptible Gram-positive and Gram-negative isolated strains had the highest frequency to enrofloxacin (82.45% and 81.81%).

Gram-positive resistant strains had the highest frequency to penicillin G (70.18%), while

Gram-negative strains had the highest frequency of resistance to lincomycin.

The results confirm the marked increase of resistance phenotypes in both Gram-positive and Gram-negative strains to a wide range of antimicrobial substances, frequently used in the therapy of infectious diseases in dogs.

In conclusion, the abusive use of antimicrobial substances cannot be recommended for the treatment of the most common respiratory tract infections in dogs, and their selection must be based on the results of susceptibility tests.

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