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RESEARCH ON THE FREQUENCY OF RESISTANCE PHENOTYPES IN BACTERIAL STRAINS ISOLATED FROM CHAMOIS (*RUPICAPRA RUPICAPRA CARPATICA*)

CERCETĂRI PRIVIND FRECVENȚA FENOTIPURILOR DE REZISTENȚĂ LA TULPINI BACTERIENE IZOLATE DE LA CAPRA NEAGRĂ (*RUPICAPRA RUPICAPRA CARPATICA*)

Iulia BUCUR[1),*)], V. DUMITRESCU[2)], K. IMRE[1)], V. HERMAN[1)], Ileana NICHITA[1)], R.T. CRISTINA[1)], E. TÎRZIU[1)]

ABSTRACT

Monitoring of circulating pathogens also in populations of wild animals is extremely important in understanding the transmission of bacterial diseases between wild and domestic animals, the epidemiological circuit between them as well as between them and humans. This research aimed at the isolation and identification of the main bacterial pathogens in the chamois (*Rupricapra rupricapra carpatica*) population in the Retezat Mountains, as well as to highlight the presence of the resistance phenotypes to strains isolated from these animals.

To isolate the bacterial strains, faecal samples from the chamois were taken and the primary identifications were made on nutrient broth. All 30 isolated strains were inseminated on chromogenic and selective media and some of them definitively identified with the Vitek 2 compact system. Based on the aspects, the isolated strains were classified into 5 bacterial species: *Enterococcus casseliflavus* (one strain), *Escherichia coli* (12 strains), *Listeria monocytogenes* (7 strains), *Salmonella spp.* (2 strains) and *Staphylococcus spp.* (8 strains). Antibiotic resistance phenotypes were determined by the Kirby-Bauer disc-diffusimetric method, using the Mueller-Hinton medium and nine antibiotic biodiscs.

By analyzing the results, it can be seen that the resistance phenotypes had a variable frequency depending on the bacterial species taken under study and also by the groups of antibiotics for which the test was carried out. The frequency of resistance phenotypes, determined by the Kirby-Bauer disc-diffusimetric method, was minimal (10%) to florfenicol and neomycin and maximum (90%) to penicillin G. All of the isolates showed very high resistance to penicillin G (90%), followed by amoxicillin with clavulanic acid and colistin sulphate (86.66%) and tetracycline (66.66%).

Keywords: antibiotic resistance, chamois, phenotypes, bacterial strains

REZUMAT

Monitorizarea agenților patogeni care circulă în populații de animale sălbatice este extrem de importantă în înțelegerea transmiterii bolilor bacteriene între animalele sălbatice și domestice, circuitul epidemiologic între acestea, precum și între ele și oameni. Această cercetare a vizat izolarea și identificarea principalilor agenți patogeni bacterieni din populația de capre negre (*Rupricapra rupricapra carpatica*) din Munții Retezat, precum și pentru a evidenția prezența fenotipurilor de rezistență la tulpinile izolate de la aceste animale.

Pentru izolarea tulpinilor bacteriene s-au prelevat probe fecale, iar identificările primare s-au făcut pe bulion. Toate cele 30 de tulpini izolate au fost însămânțate pe medii cromogene și selective, iar unele dintre ele au fost identificate cu ajutorul sistemului Vitek 2. Pe baza aspectelor, tulpinile izolate au fost clasificate în 5 specii bacteriene: *Enterococcus casseliflavus* (o tulpină), *Escherichia coli* (12 tulpini), *Listeria monocytogenes* (7 tulpini), *Salmonella* spp. (2 tulpini) și *Staphylococcus* spp. (8 tulpini). Fenotipurile de rezistență la antibiotice au fost determinate prin metoda difuzimetrică Kirby-Bauer, folosind mediul Mueller-Hinton și nouă biodiscuri cu antibiotice.

Analizând rezultatele, se poate observa că fenotipurile de rezistență au o frecvență variabilă în funcție de speciile bacteriene luate în studiu și, de asemenea, de grupurile de antibiotice la care a fost efectuat testul. Frecvența fenotipurilor de rezistență, determinată prin metoda difuzimetrică Kirby-Bauer, a fost minimă (10%) la florfenicol și neomicină și maximă (90%) la penicilină G. Toate izolatele au prezentat o rezistență foarte mare la penicilina G (90%), urmată de amoxicilină cu acid clavulanic și sulfat de colistină (86,66%) și tetraciclină (66,66%).

Cuvinte cheie: rezistență la antibiotice, capra neagră, fenotipuri, tulpini bacteriene

1) Banat's University of Agricultural Sciences and Veterinary Medicine "King Michael I of Romania", Faculty of Veterinary Medicine, Timișoara, Romania
2) Ocolul Silvic, Hațeg, Hunedoara, Romania
*) *Corresponding author: bucur_iulia@ymail.com*

Today, medical institutions save tens of thousands and even millions of human lives annually, first and foremost, with the help of antibiotics. However, almost simultaneously with the discovery of antibiotics, the

phenomenon of antimicrobial resistance also appears, which considerably reduces the possibility of using this relatively new group of medicinal remedies widely. Currently, in the United States and Canada alone, about 50% of antibiotic prescriptions for treatment cannot be justified (8, 13, 17).

Considering these circumstances and not only, several evaluation programs and strategies have been developed and implemented for the prudent consumption of antibiotics and the control over antimicrobial resistance (3, 4, 7, 8).

The phenomenon of antibioresistance to bacteria has been reported since 1950, after the introduction of sulfamides and antibiotics in the prophylaxis and therapy of infectious diseases in animals and humans. The extension of the antibiotic resistance phenomenon to Gram positive and Gram negative bacteria, pathogenic to animals and humans, has required extensive studies over time (1, 3, 7, 9, 14, 15).

Thus, the monitoring of circulating pathogens in populations of wild animals is extremely important as well in understanding the transmission of bacterial diseases between wild and domestic animals, the epidemiological circuit between them as well as between them and humans (1, 3, 6, 7, 11, 12).

This research aimed at the isolation and identification of the main bacterial pathogens in the chamois (*Rupricapra rupricapra carpatica*) population in the Retezat Mountains, as well as to highlight the presence of resistance phenotypes to strains isolated from these animals.

MATERIALS AND METHODS

In the research carried out, the frequency of resistance phenotypes of bacterial strains was emphasized. The strains were isolated and identified from the chamois (*Rupicapra rupicapra carpatica*), animals that live in natural habitats in alpine environments, respectively the area of the Retezat mountains, in Hunedoara county.

To isolate the bacterial strains, faecal samples from the chamois were taken, using at least 15 grams of faeces for each sample. For the primary inseminations made in the nutrient broth, approximately 4 grams of faeces were used for each sample, and the thermostatation was done under aerobic conditions, for 24 hours at 37°C.

Subsequently, after the bacteriostatic examination, inseminations on nutrient agar were made and the isolated strains were classified based on cultural, morphological and tinctorial characteristics. All 30 isolated strains were inseminated on chromogenic and selective media, respectively Chapman, Levine, Oxford and Rambach, and some of them definitively identified with the Vitek 2 compact system. Based on the aspects found on these environments and following the results provided by the Vitek 2 compact system, the isolated strains were classified into 5 bacterial species.

Antibiotic resistance phenotypes were determined by the Kirby-Bauer disc-diffusimetric method, using the Mueller-Hinton medium and nine antibiotic biodiscs, as follows: amoxicillin/clavulanic acid (AMC), colistin sulphate (CT), florfenicol (FFC), neomycin (N), nitrofurantoin (F), norfloxacin (NOR), penicillin G (P), streptomycin (S) and tetracycline (TE).

The results were read and interpreted according to the document European Committee on Antimicrobial Susceptibility Testing (EUCAST) and grouped in three groups, respectively susceptible strains (S), strains with intermediate behavior (I) and resistant strains (R) (19).

RESULTS AND DISCUSSIONS

As a result of the primary sowing, a total number of 30 bacterial strains were isolated from the faeces of the chamois. For the rapid identification of isolated bacterial strains, the chromogenic and selective media mentioned above were used. Also, part of them, respectively 6 strains were definitively identified using the Vitek 2 Compact system. It is mentioned that the strains selected for the definitive identification with the Vitek 2 Compact system, were the strains that on the chromogenic and selective media did not have the characteristic morphological and cultural characters.

Thus, based on these aspects, the following five bacterial species have been identified, the majority of which are important in animal pathology and contamination products of animal origin: *Enterococcus casseliflavus* (one strain), *Escherichia coli* (12 strains), *Listeria monocytogenes* (7 strains), *Salmonella spp.* (2 strains) and *Staphylococcus spp.* (8 strains).

It is mentioned that the strains identified from the chamois, were isolated from faeces, relatively fresh that came from wild chamois living in alpine environments.

Nine antibiotics, included in several classes, were used to identify resistance phenotypes, some of which were also associated with beta-lactamase inhibitors. Thus, using the phenotypic, disk-diffusimetric Kirby-Bauer method, several resistance phenotypes were detected, the number and frequency of which were variable (Table 1).

By analysing the results, it can be seen that the resistance phenotypes had a variable frequency depending on the bacterial species taken under study and also by the groups of antibiotics for which the test was carried out. Thus, the frequency of resistance phenotypes to the strains isolated from the chamois was minimal (10%) to FFC and N and maximum (90%) to P. The frequency of strains with intermediate

Table 1

Antibiogram results of strains isolated from the chamois

Crt. no.	Antibiotic	Antibiogram results						Total strains
		S		I		R		
		No.	%	No.	%	No.	%	
1.	**Amoxicillin / clavulanic acid**	1	3.33	3	10	**26**	**86.66**	**30**
2.	**Colistin sulphate**	4	13.33	0	0	**26**	**86.66**	**30**
3.	**Florfenicol**	**22**	**73.33**	5	16.66	3	10	**30**
4.	**Neomycin**	**17**	**56.66**	10	33.33	3	10	**30**
5.	**Nitrofurantoin**	8	26.66	3	10	**19**	**63.33**	**30**
6.	**Norfloxacin**	**21**	**70**	3	10	6	20	**30**
7.	**Penicillin G**	3	10	0	0	**27**	**90**	**30**
8.	**Streptomycin**	10	33.33	9	30	11	36.33	**30**
9.	**Tetracycline**	3	10	7	23.33	**20**	**66.66**	**30**

behaviour was between 10% (AMC, F and NOR) and 33.33% (N), while there were no strains with intermediate behaviour towards two antibiotics (CT and P). Susceptible strains had a frequency between 3.33% (AMC) and 73.33% (FFC).

By analysing the obtained results, it was found that the isolated bacterial species presented several resistance phenotypes, different from one species to another. Thus, in *Escherichia coli*, the most frequently isolated species (12 strains), we found most of the resistance phenotypes, respectively to all of the nine antibiotics that were used. The highest frequency (91.66 %) of the resistance phenotypes was to P, followed by the one to AMC and TE (83.33%). The frequency of *E. coli* strains with the highest intermediate behaviour was to N (50.00%), while the susceptible strains showed the highest frequency to FFC (66.66%), followed by the frequency to NOR (58.33%) (Fig. 1).

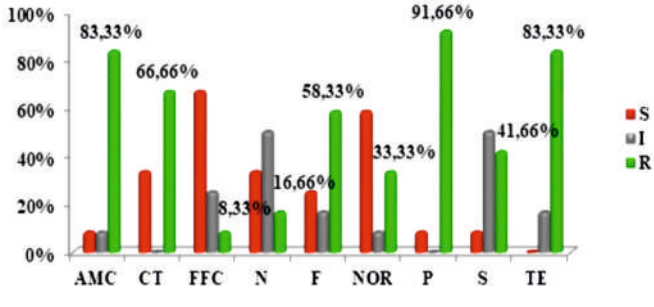

Fig. 1. Frequency of resistance phenotypes in *E. coli* strains isolated from the chamois

Also, in the case of the *Listeria monocytogenes* species, at all of the seven isolated strains, a great number of resistance phenotypes to the used antibiotics was observed. The frequency of resistance phenotypes was between 14.8% to FFC and N, respectively 100% to AMC and CT. Susceptible strains had a frequency between 14.28% to TE and 85.71% to FFC, noting that no susceptible strains were observed to AMC and CT (Fig. 2).

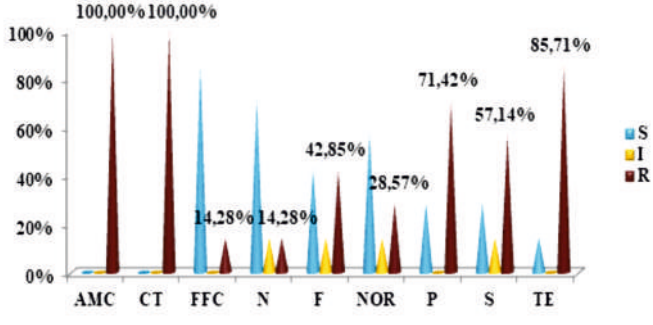

Fig. 2. Frequency of resistance phenotypes in *L. monocytogenes* strains isolated from the chamois

The resistance phenotypes, at the eight isolated strains of *Stahylococcus spp.*, were identified towards seven antibiotics, while towards N and NOR, no resistant strain was identified. Thus, the highest frequency (100%) of the resistance phenotypes was observed to AMC, CT, F and P, while the lowest frequency of 12.5% to FFC and S. The frequency of the phenotypes with intermediate behaviour was between 12.5% to NOR and 62.5% to TE. Susceptibility to isolated staphylococcus strains was observed in five of the nine antibiotics used. Thus, the frequency of susceptible strains was between 12.5% to TE and 87.5% to NOR, followed by 62.5% to FFC, N and S (Fig. 3).

The highest susceptibility (100%) for the two *Salmonella spp.* isolated strains was found towards FFC, N, F and NOR, followed by S (50%).On the other hand, the highest frequency (100%) of the resistant strains

was found to CT, P and TE, followed by the one to S (50%). At these salmonella isolated strains, no intermediate resistance strains were found (Fig. 4).

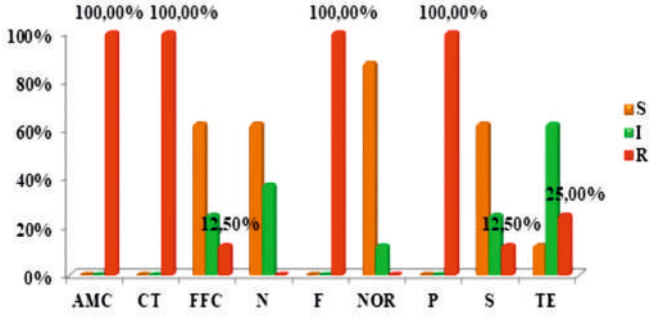

Fig. 3. Frequency of resistance phenotypes in *Staphylococcus spp.* strains isolated from the chamois

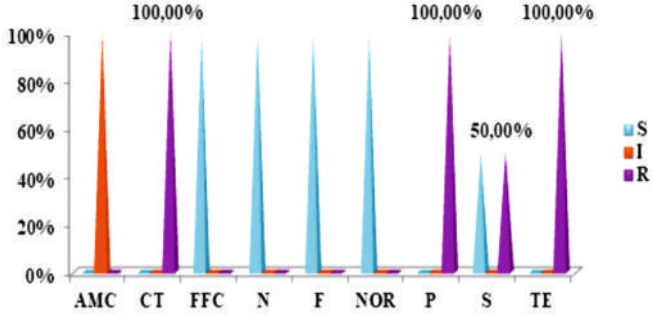

Fig. 4. Frequency of resistance phenotypes in *Salmonella spp.* strains isolated from the chamois

Resistance, in the case of the *Enterococcus casseliflavus* isolated strain, was observed towards AMC, CT, F and P, while to the other five antibiotics (FFC, N, NOR, S and TE) the strain was susceptible.

By analysing the research carried out, it was observed that, regardless of the isolated bacterial species, the resistance phenotypes were constant to CT and P, followed by the resistance phenotypes to F (with the exception of salmonella strains) and, respectively, the resistance phenotypes to S and TE (with the exception of *E. casseliflavus* strain). On the other hand, in all of the isolates the susceptible strains were observed towards FFC, N, NOR and S.

In the field of infectious pathology, identifying the resistance phenotypes at the strains isolated, both from domestic and wild animals, is an important aspect, which is why many groups of researchers study this epidemiological aspect, in many countries. The results obtained in the research, revealed the presence of the phenomenon of antibiotic resistance also at the strains isolated from wild animals, respectively the chamois (*Rupricapra rupricapra carpatica*), phenomenon noticed by other researchers (2, 5, 6, 11, 12).

Thus, in Slovakia, Vandžurová et al. (2012) studied the phenomenon of antimicrobial resistance and the presence of restriction endonucleases in faecal enterococci strains from the chamois. The authors isolated 284 strains of faecal enterococci that were tested against three antibiotics, respectively ampicillin, erythromycin and tetracycline. They identified a small frequency of resistant enterococci phenotypes and only for two of the three tested antibiotics (5% resistant phenotypes to erythromycin and tetracycline, compared to ampicillin which had no resistance phenotypes) (18).

Also in 2012, in Switzerland, Stephan and Häcler (2012) studied the antibiotic resistance phenomenon and the identification of β-lactamase producing *E. coli* strains in wild animals. For this purpose, faecal samples were taken from 84 red deer, 64 roe deer, 64 chamois and 27 ibex. From these samples, a single ESBL producing *E. coli* strain was isolated, tested on chromogenic media and definitively identified by API ID 32 E system. Further, this strain was subjected to the antibiogram and the results were the following: it was resistant to ampicillin, amoxicillin with clavulanic acid, cephalothin, cefuroxime, cefpodoxime, cefotaxime, ceftazidime, cefepime and tetracycline and susceptible to cefoxitin, imipenem, ciprofloxacin, nalidixic acid, gentamycin, streptomycin, polymyxin B and trimethoprim-sulfamethoxazole (16).

In Italy, Luzzago et al. (2014) have studied the antimicrobial resistance profile as well as virulence-associated genes in *Staphylococcus aureus* strains isolated from nasal cavities and soft tissue infections from wild ruminants. The samples were taken from two chamois and one roe deer, and the isolates of staphylococci were tested for the antimicrobial resistance to penicillin G, ampicillin, amoxicillin with clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, enrofloxacin, ciprofloxacin, tetracycline, doxycycline, trimethoprim-sulfamethoxazole, erythromycin, streptomycin, kanamycin, gentamicin and tobramycin. Only one chamois isolate showed resistance to penicillin, ampicillin, amoxycillin with clavulanic acid, cefoxitin, ceftiofur, ceftriaxone, ciprofloxacin and enrofloxacin, and the methicillin resistance was confirmed by the presence of the *mec*A gene, performed by PCR techniques (10).

CONCLUSIONS

Based on the morphocultural characteristics observed on selective/chromogenic media and with the identification using the Vitek 2 Compact system, 30 bacterial strains from the chamois faeces were isolated, of which 12 belonged to the *E.coli* species, seven to the *Listeria monocytogenes* species, two of the genus *Salmonella*, eight of the genus *Staphylococcus* and one strain of *Enterococcus casseliflavus*. The frequency of resistance phenotypes, determined by the Kirby-Bauer disc-diffusimetric method, was minimal (10%) to florfenicol and neomycin and maximum (90%) to penicillin

G. All of the isolates showed very high resistance to penicillin G (90%), followed by amoxicillin with clavulanic acid and colistin sulphate (86.66 %) and tetracycline (66.66%).

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