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PREVALENCE AND CHARACTERISATION OF EXTENDED-SPECTRUM BETA-LACTAMASES AND PLASMID-MEDIATED QUINOLONES RESISTANCE IN *Enterobacteriaceae* ISOLATED FROM COMPANION ANIMALS

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ABSTRACT. Antimicrobial resistance is a major public health concern worldwide. This study aims to determine the prevalence of *Enterobacterales* producing beta-lactamase (TEM, SHV, OXA) or extended-spectrum beta-lactamases (ESBL), as well as plasmid-mediated resistance to quinolones (PMQR) (*qnrA, qnrB, qnrS*) in companion animals from the northeast region of Romania. A total of 124 faecal samples were collected aseptically from healthy dogs attending the veterinary practice for vaccination and cultivated on Brilliance ESBL medium (Oxoid, UK). The ESBL production testing was performed using the combination disc test. The identification of *Enterobacterales* strains was achieved using molecular identification and based on biochemical tests. Antimicrobial susceptibility testing was performed using the disk diffusion method. Identification of genes encoding for beta-lactamase enzymes and genes encoding plasmid-mediated resistance to quinolones was performed by PCR according to the protocols previously described. After ESBL screening, 31 (31/124; 25%) extended-spectrum cephalosporin (ESC)-resistant *Enterobacterales* were obtained, and 67.74%

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(21/31) of them were confirmed as ESBL-producers. Regarding the *Enterobacterales* species, 27 (27/31; 87.1%) were *Escherichia coli* and 4 (4/31; 12.9%) strains were *Klebsiella pneumoniae*. Among the ESBL-producing isolates, the $bla_{\text{CTX-M-1}}$ gene group was predominant (58.82%), followed by the $bla_{\text{CTX-M-9}}$ group (41.18%). The bla_{TEM}, bla_{SHV} and bla_{OXA} gene groups were identified in 54.83%, 29.03% and 3.22% of the analysed strains, respectively. The prevalence of PMQR genes was 22.58% and consisted only of *qnrS* (19.35%) and *qnrA* (3.22%) genes. The prevalence of ESBL strains related to the total number of analysed samples was 16.93% (21/124). The findings show a significant prevalence of ESBLs and PMQR genes in *Enterobacterales* strains isolated from the faeces of healthy dogs, implying that pets may pose a risk of transmitting ESBL strains to other animals or owners.

Keywords: antimicrobial resistance; companion animals; ESBL genes.

INTRODUCTION

Antimicrobial resistance (AMR) is a major public health issue affecting both human and veterinary medicine. Extended-spectrum beta-lactamase (ESBL) enzymes provide resistance to third and fourth-generation cephalosporins and also to aztreonam, the newest antibiotic available to treat enterobacteria infections, such as *Escherichia coli* or *Klebsiella pneumoniae* (Bush and Jacoby, 2010). Research on extended-spectrum cephalosporin (ESC)-resistant *Enterobacteriaceae* strains isolated from pets has increased over the past 20 years.

However, the prevalence of these strains in some countries, especially in less developed countries, has not been reported. Furthermore, although antibiotic use in domestic animals is widespread, relatively few studies have quantified antibiotic usage and AMR in dogs and cats compared to the research in other species or categories of animals and humans. It is known that very close daily contact with humans is an important risk factor for transmission of these strains within or between species.

The aim of this study was to determine the prevalence of *Enterobacteriaceae* strains that produce beta-lactamase enzymes (TEM, SHV, OXA), ESBL, and genes encoding plasmid-mediated resistance to quinolones (PMQR) (*qnrA, qnrB, qnrS*).

MATERIALS AND METHODS

Faecal samples were collected aseptically using rectal swabs from clinically healthy dogs that came to the veterinary clinic for vaccination. After collection, the samples were immediately inoculated onto the screening medium Brilliance ESBL (Oxoid, Basingstoke, UK), as described in a previous article by the authors (Cozma *et al.*, 2019).

The bacterial strains that showed characteristic *Enterobacteriaceae* colonies on the screening medium were subcultured onto blood agar medium (Oxoid, Basingstoke, UK) for subsequent testing and identification. For *E. coli* isolates, species confirmation was performed by PCR based on molecular identification of the *uidA* and *uspA* genes (Anastasi *et al.*, 2010; McDaniels *et al.*, 1996) and for the *K. pneumoniae* isolates, based on biochemical tests (API 20E, Biomerieux, Marcy-l'Étoile, France).

Phenotypic characterisation of ESBL production was performed for all

isolated strains (n = 31). The colonies identified as *E. coli* and *K. pneumoniae* were subcultured onto blood agar medium and tested using the combination disc test (Cozma *et al.*, 2019). All isolates (n = 31) were also antibiotic susceptibility tested using the disk diffusion method on Muller–Hinton agar medium.

The data were interpreted in conformity with the Clinical and Laboratory Standards Institute (CLSI) recommendations (CLSI, 2018). If an isolate had intermediate or resistant results against the tested antimicrobial agent, it was considered non-susceptible.

The following antibiotics were included in the antimicrobial panel: ampicillin (10µg), amoxicillin/clavulanic acid (30µg), imipenem (10µg), aztreonam (30µg), enrofloxacin (5µg), trimethoprim/ sulfamethoxazole (25µg), tetracycline (30µg), chloramphenicol (30µg), and gentamicin (10µg). One ATCC standard strain of *E. coli* (*E. coli* ATCC 25,922) was used as a control strain.

Genetic background characterisation was done by PCR for the genes that produce beta-lactamase enzymes, ESBL and PMQR. The extraction of bacterial DNA was performed using the boiled preps method (Maciuca *et al.*, 2015). By using PCR according to the previously described protocols, we aimed to identify the gene groups $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and the PMQR genes, respectively (Dallenne *et al.*, 2010; Robicsek *et al.*, 2006; Wedley *et al.*, 2011)

RESULTS

A total of 124 faecal samples were collected aseptically from clinically healthy dogs that came to the veterinary clinic for vaccination. Following ESBL screening, 31 (31/124; 25%) *Enterobacteriaceae* strains resistant to extended cephalosporins were obtained, of which 27 (27/31; 87.1%) were *E. coli*, and 4 (4/31; 12.9%) strains were *K. pneumoniae*.

All 31 extended-spectrum cephalosporin-resistant (ESC-R) *Enterobacterales* were tested for antimicrobial susceptibility. All organisms were resistant to ampicillin, with 77.41% resistant to amoxicillin/ clavulanic acid, 61.29% resistant to sulfamethoxazole/trimethoprim, 58.06% resistant to tetracycline, and 45.16% resistant to chloramphenicol, gentamicin and enrofloxacin (*Figure 1*).

All isolated strains were analysed to assess the degree of multidrug resistance (MDR). According to Magiorakos *et al.* (2012), resistance to more than three classes of antibiotics defines a strain as being MDR. Following the analysis of the results obtained in the disk diffusion antibiotic susceptibility test, 18 (18/31; 58.06%) strains were associated with MDR.

Molecular characterisation of the genetic background was done for the 31 ESC-R *Enterobacterales* carrying genes or gene combinations (*Table 1*). The most prevalent groups of genes were the $bla_{\text{CTX-M}}$ gene group (54.83%; 17/31) and the bla_{TEM} gene group (54.83%; 17/31) (*Figure 2*). In the $bla_{\text{CTX-M}}$ gene group, the $bla_{\text{CTX-M-1}}$ group was identified as predominant (10/17; 58.82%), along with the $bla_{\text{CTX-M-9}}$ group (7/17; 41.18%) (*Figure 2*).

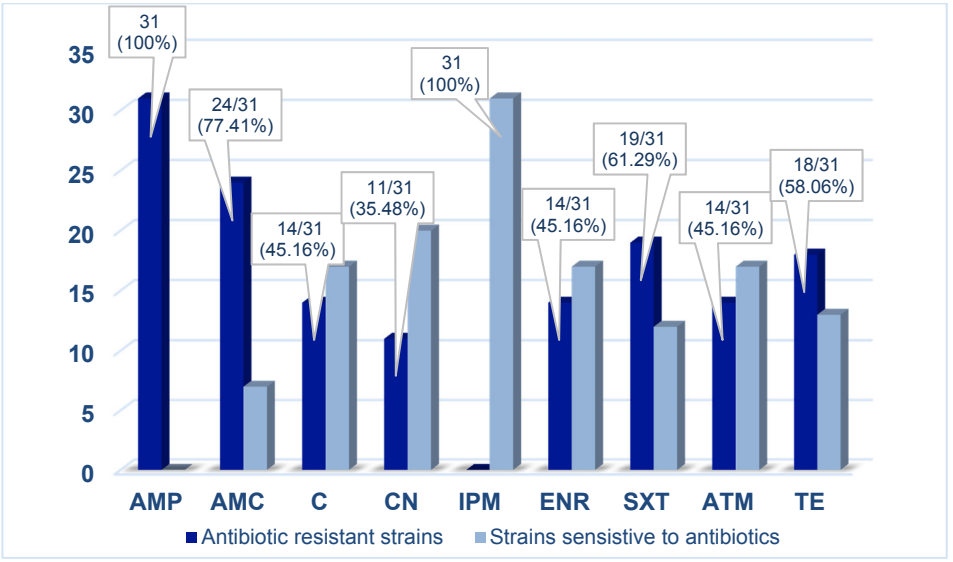

Figure 1 – Antimicrobial susceptibility testing results (n = 31 isolates)
Abbreviations: AMP, ampicillin; AMC, amoxicillin/clavulanic acid;
C, chloramphenicol; CN, gentamicin; IPM, imipenem; ENR, enrofloxacin;
SXT, trimethoprim/sulfamethoxazole; ATM, aztreonam; TE, tetracycline

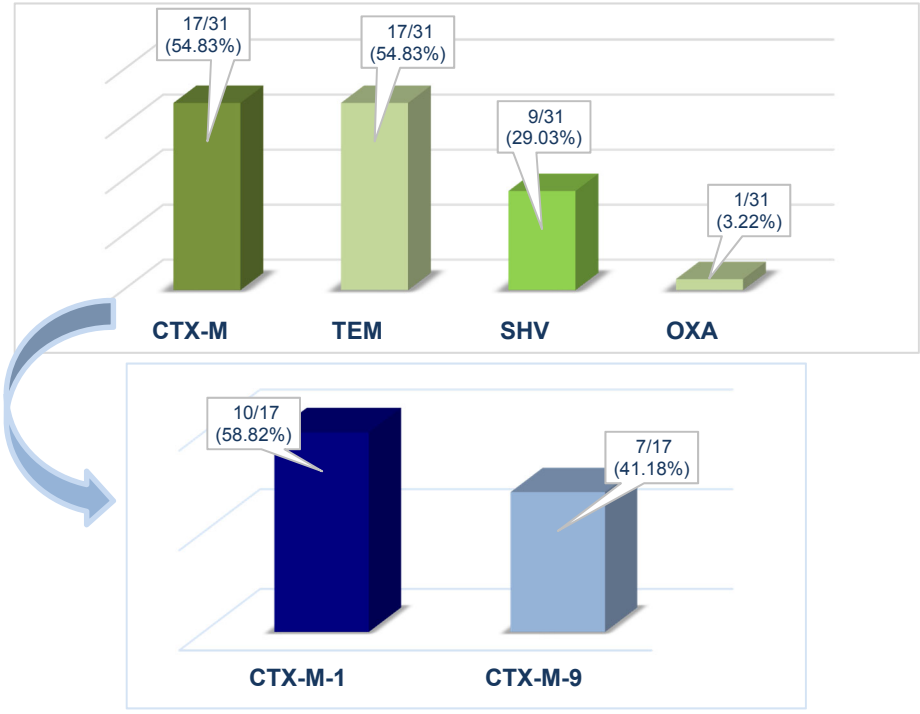

Figure 2 – Molecular characterisation of the genetic background regarding antibiotic resistance of the isolated strains

The *bla*$_{SHV}$ and *bla*$_{OXA}$ gene groups were identified in 9/31 (29.03%) and 1/31 (3.22%), respectively, of the analysed strains (*Figure 2*). In some isolates, only the *bla*$_{TEM}$ gene (6/31; 19.35%), the *bla*$_{SHV}$ gene (4/31; 12.9%), or combinations of the *bla*$_{TEM}$ and *bla*$_{SHV}$ (3/31; 9.68%) were present, and only one strain (1/31; 3.22%) had the genes *bla*$_{TEM}$, *bla*$_{SHV}$ and *bla*$_{OXA}$ (*Table 1*). The prevalence of PMQR genes was 22.58% (7/31), and only the *qnrS* (6/31; 19.35%) and *qnrA* (1/3; 3.22%) genes were identified (*Figure 3*).

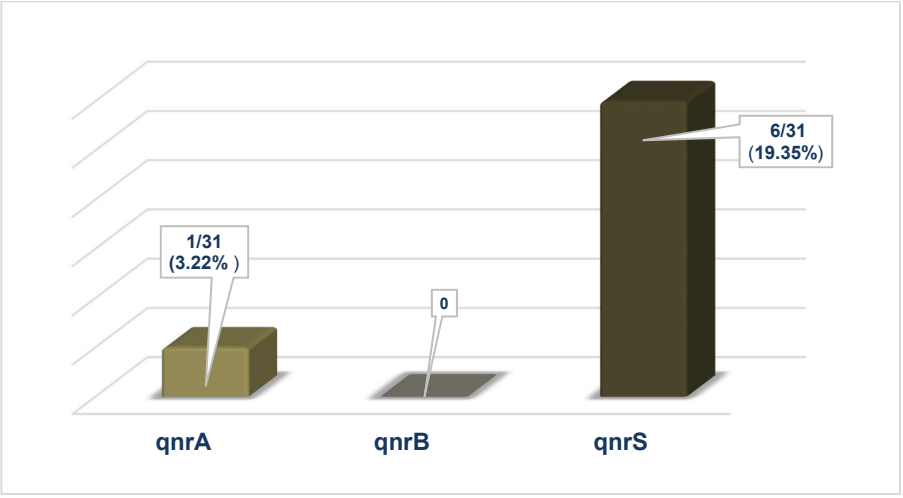

Figure 3 – Molecular characterisation of the genetic background regarding plasmid-mediated resistance to quinolones

Table 1 – Molecular identification of extended-spectrum cephalosporin-resistant (ESC-R) *Enterobacterales* in dog faeces

Bacterial species	No. and isolate ID	ESBL phenotype YES/NO	Beta-lactamase genes by PCR	Associated PMQR genes
E. coli	3 (MV67;MV46;MV67i)	YES	blaCTX-M-1 group	-
	5 (MV18;MV19;MV22;MV21;MV40)	YES	blaCTX-M-1 group	qnrS
	1 (MV73)	YES	blaCTX-M-1 group; blaTEM	qnrS
	1 (MV47)	YES	blaCTX-M-1 group; blaTEM; blaSHV	-
	2 (MV39;MV43)	YES	blaCTX-M-9 group	-
	5 (MV20;MV66;MV16;MV66p;MV17)	YES	blaCTX-M-9 group; blaTEM	-
	1 (MV37)	YES	blaTEM	-
	5 (MV29; MV30; MV33; MV62; MV63)	NO	blaTEM	-
	1 (MV38)	YES	blaTEM; blaSHV	-
	1 (MV34)	YES	blaTEM; blaSHV	qnrA
	1 (MV31)	YES	blaTEM; blaSHV; blaOXA	-
	1 (MV71)	NO	blaTEM; blaSHV;	-
K. pneumoniae	14 (MV71p; MV71K; MV68E; MV68)	NO	blaSHV	-

The strains carrying *bla*$_{CTX-M}$ genes are considered ESBL enzyme-producing strains (Zeynudin *et al.*, 2018). The *bla*$_{TEM}$, *bla*$_{SHV}$ or *bla*$_{OXA}$ gene groups encode both beta-lactamase and ESBL enzymes (Ewers *et al.*, 2011). In this study, the strains that were carriers of the *bla*$_{TEM}$, *bla*$_{SHV}$ or *bla*$_{OXA}$ genes were confirmed as being ESBL-producing strains correlating the molecular results with the results obtained in the combination disc method.

Therefore, the prevalence of ESBL strains, of the 31 strains resistant to extended cephalosporins, was 67.74% (21/31). The prevalence of ESBL strains related to the total number of analysed samples was 16.93% (21/124).

DISCUSSION

In Romania, there are more publications on the prevalence of AMR in human bacterial isolates (ECDC, 2022), but for veterinary medicine, the data are limited. This study has analysed bacterial phenotypes in combination with the genetic characteristics of *E. coli* and *K. pneumoniae* strains isolated from healthy dogs from the northeast region of Romania.

The 16.93% prevalence of ESBL strains in this study is much higher than the global average (6.87%) identified in dogs (Salgado-Caxito *et al.*, 2021) and similar to the prevalence obtained in strains isolated from chicken in Romania (Maciuca *et al.*, 2015). Also, compared to other similar studies carried out in Europe, the obtained prevalence is lower than in countries such as France (18.5%) or Spain (19.6%) (Abreu-Salinas *et al.*, 2020; Haenni *et al.*, 2014).

The *bla*$_{CTX-M}$ genotype was most commonly identified among the isolated strains and was reported in approximately 95% of similarly conducted studies (Salgado-Caxito *et al.*, 2021). Moreover, the *bla*$_{CTX-M-1}$ genotype is the most commonly identified in similar studies. In addition, like in similar studies, our study showed that the *qnr* gene family, *qnrs,* was the most prevalent. Moreover, the PMQR variants (*qnrS* and *qnrA*) were coexpressed with the beta-lactamase or ESBL enzymes (Cui *et al.*, 2022; de Jong *et al.*, 2018).

The World Health Organization (WHO) has stated that quinolone antibiotics, beta-lactam antibiotics (such as third, fourth and fifth-generation cephalosporins, and aminopenicillins with and without beta-lactamase inhibitors) and aminoglycosides are antibiotics of critical importance (WHO, 2007).

All of the above-mentioned antibiotics are also used in veterinary medicine and this study has shown increased resistance to some of the tested antibiotics of critical importance.

The percentage of antibiotic-resistant strains varies by country. Antibiotic resistance is also highly dependent on the implementation of public policies regulating antibiotic prescribing, especially in veterinary medicine, which has been shown to be related to the emergence of MDR strains.

Moreover, the use of antibiotics in animal feed, without quantification or with empirical dosing, and the preferential use of enrofloxacin and doxycycline without alternating them

with other antibiotics were other factors influencing the emergence of MDR strains in veterinary medicine (Dierikx *et al.*, 2012).

CONCLUSIONS

Researchers discovered a high incidence of ESBLs in *Enterobacteriaceae* strains isolated from the faeces of clinically healthy companion animals (dogs), indicating the risk of ESBL strains spreading to other animals or owners.

Research on ESC-resistant *Enterobacteriaceae* in companion animals has increased in recent years, showing that these bacteria are present in dogs worldwide. However, their prevalence in some companion animal populations from many countries, including Romania, has not yet been reported; this is concerning given the shared environment and close contact with the owners.

Future research should focus on the identification of the factors responsible for the acquisition and dissemination of ESC-resistant *Enterobacteriaceae* in pets, including interspecies transmission, clinical relevance and their economic impact.

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