

Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

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spp. in companion animals and calves and prevalence of
Campylobacter spp. in companion animals**

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Orientador: Prof. Doutor Paulo Martins da Costa

Coorientadora: Dr.^a Marisa Isabel Ribeiro de Almeida

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Abstract

The human-animal bond has developed and shifted throughout the years, with unarguably significant impacts in the health and living conditions of both populations, that are continuously growing and expanding into new geographic areas. Indeed, animals and humans share the same eco-systems and could be infected by the same microbes. Keeping in mind this One Health perspective, in which human, animal and environmental health are intrinsically connected, this study focused mainly on the research of colistin resistant bacteria in companion animals and bovines, as well as on the detection of *Campylobacter* spp. among pets. Regarding companion animals, it was possible to recover both colistin resistant *E. coli* isolates and *Campylobacter jejuni* and *C. upsaliensis* in three and six out of 50 rectal swabs obtained from dogs and cats housed in a shelter (n=16) or presented to consultation at the Veterinary Hospital of the University of Porto (n=34). Also, within this group, an extended spectrum beta-lactamase producing *E. coli* was recovered, as well as four isolates presenting a multi-drug resistant profile. In what concerns bovines, colistin resistant isolates were recovered from a milk sample (7%), albeit displaying a lower prevalence when compared to pets; within this group, six beta-lactamase producing isolates were recovered. Therefore, it was possible to detect, in all sampled populations, the presence of potentially harmful bacteria; the characteristics of the recovered bacteria make them a worrisome threat for public health. These findings further highlight the importance of continuous studying and monitoring on this field of research, in order to take adequate action and increase awareness on its importance.

Resumo

A interação entre humanos e animais tem vindo a evoluir ao longo das últimas décadas. Ambas as populações têm crescido em número, em ocupação territorial e em interdependência, acarretando impactos indeléveis na saúde e bem-estar. Com base na perspetiva One Health – segundo a qual a saúde humana, animal e do meio ambiente são indissociáveis – o presente estudo centrou-se em particular na pesquisa de bactérias resistentes a colistina em animais de companhia e em bovinos, bem como na pesquisa de *Campylobacter* spp. em cães e gatos. No decurso deste trabalho de investigação, foi possível isolar tanto *E. coli* resistente a colistina como *C. jejuni* e *C. upsaliensis* em três e em seis amostras, respetivamente, num total de 50 zaragatoas retais recolhidas em animais de companhia alojados em Centro de Recolha Oficial (n=16) ou apresentados a consulta no Hospital Veterinário da Universidade do Porto. (n=34). Neste grupo foi também possível detetar um isolado de *E. coli* produtor de beta-lactamases, bem como quatro isolados com perfil de multirresistência. Relativamente aos bovinos, isolou-se uma estirpe de *E. coli* resistente a colistina (7%) numa amostra de leite fornecido aos vitelos. Deste grupo obtiveram-se ainda seis isolados produtores de beta-lactamases. Assim, foi possível detetar, em todos os grupos em estudo, a presença de bactérias potencialmente patogénicas e/ou capazes de expressar fenótipos de resistência preocupantes para a saúde humana e animal. Estes achados evidenciam, assim, a importância fulcral do estudo e monitorização contínuos nesta área de estudo, de modo a tomar medidas adequadas e atempadas, e aumentar o conhecimento geral nestes assuntos.

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Abbreviations

AMR	Antimicrobial Resistance
BA	Blood Agar
BPW	Buffered Peptone Water
BHI	Brain Heart Infusion
CLSI	Clinical Laboratory Standards Institute
Col	Colistin
EUCAST	European Committee for Antimicrobial Susceptibility Testing
ESBL	Extended Spectrum Beta Lactamase
HEA	Hektoen Enteric Agar
LPS	Lipopolysaccharide
MDR	Multidrug Resistant
MH	Mueller-Hinton Agar
MHB2	Cation-adjusted Mueller-Hinton Agar
MIC	Minimal Inhibitory Concentration
MIU	Motility/Indole/Urease Agar
ORBEA	Organismo Responsável pelo Bem-Estar Animal do ICBAS – UP
SCAi + Col	Simmons Citrate Agar + 1% myo-inositol + Colistin
SCAi	Simmons Citrate Agar + 1% myo-inositol
TBX + Col	Tryptone Bile X-glucuronide Agar + Colistin
TBX	Tryptone Bile X-glucuronide Agar
TSB	Trypto-casein Soy Broth
TSI	Triple Sugar Iron slant

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Rationale

When faced with a modern-day society that relates closely to and depends on animals, their products, and the environment, it is of the utmost importance to keep in mind the interactions of these parts, and how changes happening on one of these sectors will, inevitably, impact the others. The awareness and acknowledgment of the importance of these interactions are the cornerstone of the One Health concept, that advocates an interdisciplinary approach to the human, animal and environmental health, promoting collaboration between various branches of knowledge to reach common goals (Chaddock, 2012; Zinsstag *et al.*, 2020).

Undeniably, the ever-evolving and changing Man – Animal bond affects humans in several ways. Despite having a positive impact on human health, these animals can impact the environment around them and be an important source of harmful organisms. Most pets share living spaces in close proximity with their owners, being included in diverse social settings. Moreover, with increasing and improved veterinary care, animals tend to live longer and reach geriatric ages, when therapy with antimicrobials may be required more often (Marchetti *et al.*, 2021; Overgaauw *et al.*, 2020).

As antimicrobial resistance (AMR) affects both human and animal health, and considering an ever growing and globalized human population, which relies increasingly on healthy animal populations, a collaborative and multi-disciplinary strategy is urgently needed (Léger *et al.*, 2018; Naylor *et al.*, 2021). According to data published by the European Centre for Disease Prevention and Control, over 670.000 bacterial infections are caused by MDR, 33.000 of which result in fatal disease (Binsker *et al.*, 2022).

Bacteria are able to make use of their evolutionary mechanisms and genomic flexibility to protect themselves from harmful substances. In addition, bacteria possess efficient mechanisms to spread these resistance mechanisms, such plasmid-induced resistance, that represents a big portion of bacterial resistances (Palma *et al.*, 2020). The veterinary practice is considered to have an important role in the appearance of AMR, with overuse or misuse of antimicrobial drugs, particularly within livestock (Ferriol-González & Domingo-Calap, 2021; Lhermie *et al.*, 2017; World Organisation for Animal Health (OIE), 2021).

One particular example of this is the existence of colistin resistant *Enterobacteriaceae*. Also known as polymyxin E, colistin was discovered in 1947, being one of the first antimicrobials with activity against Gram-negative bacteria. Although its use in human medicine was abandoned for a long time, due to concerns about the possible nephrotoxic and neurotoxic effects, its use in veterinary medicine remained widely spread, particularly in swine and cattle, either as growth promoter, metaphylaxis/prophylaxis, or individual treatment. However, the worrying rise of multi-

drug resistant (MDR) Gram-negatives, including *Enterobacteriaceae*, has placed colistin, in many situations, as a last-resort drug against infections caused by these organisms (Kempf *et al.*, 2016; Kieffer *et al.*, 2014; Nation & Li, 2009).

Resistance to colistin seems to be associated primarily with structural changes to the lipopolysaccharide (LPS) of the bacterial wall; these changes occur mainly on the lipid A, reducing the electrostatic interactions with colistin. (Lima *et al.*, 2019). These modifications can be attributed either to a chromosome-encoded mechanism, due to mutations in *pmrAB*, *phoPQ* and *mgrB* genes (Joshi *et al.*, 2019), or a mechanism of resistance associated with mobile genetic elements, the *mcr* gene, in which the addition of phosphoethanolamine to lipid A dampens the net negative charge and consequently reduces the affinity of colistin to lipid A. Ever since it was first detected, *mcr-1* and other similar variants have been increasingly reported across five different continents. Moreover, the presence of *mcr* genes in plasmids enables colistin resistance dissemination by mechanisms of horizontal gene transfer, such as conjugation, making it possible for gut colonizing organisms to exchange genetic material among each other, whether the involved bacteria are commensal or pathogenic (Lima *et al.*, 2019; Luo *et al.*, 2020). Families of bacteria such as *Enterobacteriaceae* comprise a large number of bacterial species, such as *Escherichia coli* and *Klebsiella pneumoniae* (Fam *et al.*, 2015), that could be characterized as commensal or pathogenic bacteria and can be found in animals, humans and in a wide diversity of environments (Bista *et al.*, 2020).

Pathogenic and zoonotic microorganisms have their transmission potential increased by a shared environment and close proximity between animals and humans. Data reveals that 60% of pathogens responsible for disease in human have their origin in domestic animals or wildlife, and 75% of the emerging human pathogens are of animal origin (World Organisation for Animal Health-OIE, 2021b). *Campylobacter* spp., a zoonotic pathogen, is considered one of the four most important global causes of diarrheal conditions, sometimes leading to life-threatening disease; it is widely distributed in warm-blooded animals, including in pets. The most relevant route of transmission is believed to be foodborne, via contaminated meat or milk (World Health Organization, 2020). However, other sources of infection have been described, such as wild animals, and pets. Owning a dog has been described as a risk factor for *Campylobacteriosis*, and a percentage of human cases of the disease have been attributed to companion animals (Lemos *et al.*, 2021).

Chapter One

Colistin resistant *E. coli* and *Klebsiella* spp. in Companion Animals

1. Introduction

Over the past decades, the relationship between companion animals and humans has evolved; what was initially based on a primarily cooperative relationship (e.g. hunting and guard dogs) morphed into companionship and a closer cohabitation inside the same house (Overgaauw *et al.*, 2020). While this bond has an undeniably positive effect on human health and, oftentimes, also assures these animals receive adequate veterinary care, the close proximity increases the risk of bacterial spread and transmission between pets and owners (Marchetti *et al.*, 2021; Overgaauw *et al.*, 2020).

Members of the Enterobacteriaceae family are commonly found in a variety of environments, both in humans and animals, and possess the ability to cause disease. Bacteria such as *Escherichia coli* and *Klebsiella* spp. are included in this group, presenting a great variety of hosts (Bista *et al.*, 2020). The former can be found in the normal gut flora of humans and worm blooded animals and can cause, under specific circumstances, severe disease (Bista *et al.*, 2020). *Klebsiella* spp. are also Gram-negative bacilli, that can be present on a diversity of environments, including mucosal surfaces of animals and humans. They are responsible for several infections, both in companion animals and humans (Harada *et al.*, 2016).

Colistin is a last-resource drug against Enterobacteriaceae infections; thus, *E. coli* and *Klebsiella* spp. -resistant strains are challenging for healthcare services. Most colistin products available in the European Union (EU) are authorized for food-producing animals (e.g., pigs, broilers, beef cattle) but a very reduced number of formulations for use in companion animals is available and authorized, resulting in an infrequent use of this drug within this group. Nonetheless, resistance to colistin in pets has been reported, being the environment the most probable source (Hamame *et al.*, 2022).

The presence of the *mcr* gene, encoding resistance to colistin, has been reported in both bacteria mentioned above. However, *E. coli* - due to the variety of hosts and environments in which it can be found - is considered the main host of *mcr-1* genes (Chandler *et al.*, 2020), being represented in over 90% of the *mcr*-positive isolates reported. The *mcr* genes 1, 2, 3, 7, 8 and 9 have been identified in various strains of *Klebsiella* spp., with several studies describing a much higher *mcr* isolation rate in animals when compared with human clinical data (Luo *et al.*, 2020).

While information on the prevalence of colistin resistance among companion animals is scarce (Hamame *et al.*, 2022), their closeness to humans and their potential

for carrying and/or spreading MDR bacteria poses a threat to human health and requires investigation and monitorization.

1.1. Aim of the study

The purpose of this work was to detect the presence, and to perform the phenotypical characterization, including antimicrobial susceptibility and genotypic characterization, of colistin resistant and *mcr*-positive *E. coli* and *Klebsiella* spp. isolated in dogs and cats.

1.2. Materials and Methods

1.2.1. Sampling

To assess the prevalence of colistin resistant *E. coli* and *Klebsiella* spp., two groups were established, comprising a total of 50 samples. The first group (group S – Shelter), was comprised of 16 rectal swabs, obtained from dogs (n=12) and cats (n=4) housed in a shelter, located in Coimbra, Portugal. The second group (group H - Hospital) consisted of 34 rectal swabs obtained from dogs (n= 24) and cats (n = 10) presented to consultation at the Veterinary Hospital of the University of Porto (UPVet), between December 27th, 2021, and February 14th, 2022. The samples were selected among the animals presented for consultation and/or treatment. For both groups in study, a written consent was provided to the persons in charge and information was gathered on the animals' general living conditions, dietary habits, and recent history of antimicrobial treatment (Annex A and B). This study had the approval of the Organismo Responsável pelo Bem-estar Animal do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto (ORBEA ICBAS-UP).

1.2.2. Culture and isolation of *E. coli* and *Klebsiella* spp.

Immediately after performing the rectal swab, the sample was placed in 5mL of Buffered Peptone Water (BPW) (Liofilchem, Teramo, Italy) and left for one hour at room temperature. After this revitalization step, 100 µL of this suspension was spread in (i) Tryptone Bile X-glucuronide Agar (TBX) (Liofilchem) and (ii) TBX + 3,5 mg/L of colistin, for the detection of typical *E. coli* colonies (International Organization for Standardization, 2015), and also on (iii) Simmons Citrate Agar + 1% myo-inositol + 3,5 mg/L of colistin (SCAi) (Liofilchem), for the detection of typical *Klebsiella* spp. colonies (Van Kregten *et al.*, 1984). TBX and TBX + Col medium were left to incubate for 24h at 37°C, while SCAi + Col medium was left to incubate for 48h at 37°C. A volume of 10 µL of this suspension were also streaked on simple TBX medium and left to incubate at 37°C for 24h. The

remaining suspension was left to incubate at 37°C for 18 hours; then, 100 µL were spread in TBX + Col and SCAi + Col; these selective and enriched culture media were incubated at 37°C for 24h and 48h, respectively.

In addition to this, 10 µL of post-enrichment were inoculated in a previously prepared 5 mL of Brilliant Green Bile Broth (ThermoScientific, Massachusetts, USA), enriched with 3,5 mg/L of colistin and incubated for 24h at 44,5°C. If bacterial growth was observed (turbidity) after the incubation period, 1 µL of the broth was spread in TBX agar.

1.2.3. Identification of isolates

Colonies growing on TBX + Col, SCAi + Col and TBX (coming from Green Bile Broth) with typical *E. coli* or *Klebsiella* spp. appearance were then subcultured in Hektoen Enteric Agar - HEA (Liofilchem). Biochemical tests were performed on Motility-Indole-Urease Agar (MIU) (Liofilchem), Triple Sugar Iron slant (TSI) (Liofilchem) and Simmons citrate (Liofilchem) and the results were interpreted using the tables provided in *Bergey's Manual of Determinative Bacteriology* (American Society of Microbiology *et al.*, 1957). Colonies identified as *E. coli* or *Klebsiella* spp. were frozen in Tryptic Soy broth (TSB) + 1,5% glycerol (Biokar Diagnostics) at -20°C.

DNA was extracted from pure colonies, by suspending a colony in 20 µL TE buffer (Tris 10 mM + EDTA 1 mM, pH = 8), which was then incubated for 15 minutes at 95°C, followed by the addition of 180 µL of sterile ultrapure water; the mixture was centrifuged at 12 000 rpm for 3 minutes and the supernatant was preserved at -20°C.

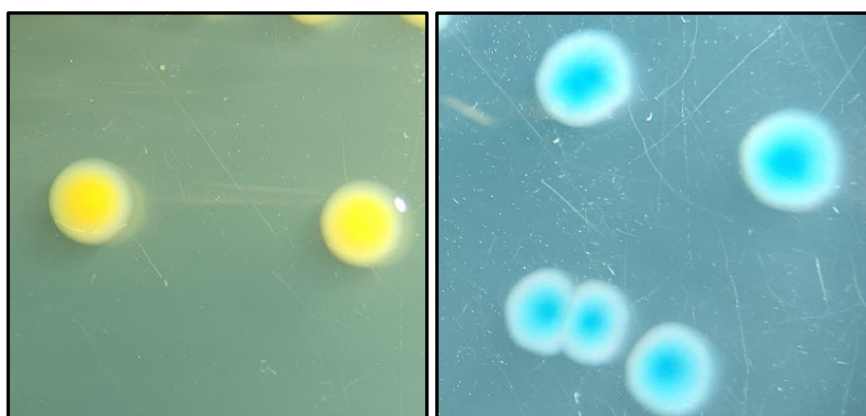


Figure 1. Typical *Klebsiella* spp. appearance on SCAi medium (left) and typical *E. coli* appearance on TBX medium (right)

1.2.4. Antimicrobial susceptibility testing

At least two isolates from each culture media: TBX, TBX + Col, SCAi + Col and Green Bile Broth spread on TBX were selected on the basis of colony size and morphology. Susceptibility to antimicrobial agents was tested using the disk diffusion method according to the National Committee for Clinical Laboratory Standards Guidelines (Clinical and Laboratory Standards Institute, 2018b), using the following antimicrobial drugs (Oxoid): Ampicillin (AMP) (10 µg), Amoxicillin-Clavulanate (AMC) (30 µg), Cefazoline (KZ) (30 µg), Ceftazidime (CAZ) (30 µg), Cefoxitin (FOX) (30 µg), Cefotaxime (CTX) (30 µg), Aztreonam (ATM) (30 µg), Gentamycin (CN) (10 µg), Tobramycin (TOB) (10 µg), Amikacin (AK) (30 µg), Streptomycin (S) (10 µg), Tetracycline (TE) (30 µg), Doxycycline (DO) (30 µg), Ciprofloxacin (CIP) (5 µg), Levofloxacin (LEV) (5 µg), Sulfamethoxazole-Trimethoprim (SXT) (25 µg), Chloramphenicol (C) (30 µg), Nitrofurantoin (F) (300 µg), and Imipenem (IPM) (10 µg).

1.2.5. Colistin susceptibility testing – Broth Microdilution Method

Broth microdilution, the gold standard for colistin resistance testing, (*National Programme on Antimicrobial Resistance Containment National Centre for Disease Control*, 2020; Sun *et al.*, 2018), was performed on every *E. coli* and *Klebsiella* spp., recovered from selective media with colistin. According to CLSI criteria, an isolate is considered colistin-resistant if it shows a Minimal Inhibitory Concentration (MIC_{Col}) of at least 4 µg/mL, *i.e.*, when the bacterial suspension is placed through increasing concentrations of colistin, the microplate well containing a concentration of 4 µg/mL is the first well to display no growth. Isolates displaying a MIC_{Col} of 2 µg/mL can be considered to have intermediate resistance (CLSI, 2020).

To perform this test, different concentrations of colistin (1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml and 16 µg/ml) were placed in a 96-well plate, each well containing 50 µL of Cation-adjusted Mueller-Hinton Broth (MHB2) (Sigma–Aldrich, Missouri, USA). Bacterial cultures tested were previously grown in MH (37°C for 18h). Colonies were suspended on 9 mL of MHB2 and adjusted to a 0,5 MacFarland turbidity; then, 50 µL of the suspension were then transferred to the microplate, in order to test different colistin concentrations for each isolate, with a final number of bacteria per well of approximately 5×10^5 CFU/mL. Control of suspension and control of medium were performed in each microplate.

Finally, microplates were incubated for 20h and results were read (Clinical and Laboratory Standards Institute, 2018; European Committee on antimicrobial susceptibility testing - EUCAST, 2020).

1.2.6. PCR for detection of *mcr* genes

The detection of *mcr* genes was performed on all isolates recovered from selective media with colistin using a Multiplex Polymerase Chain Reaction (PCR) targeting *mcr* 1 to 5 and *mcr* 6 to 9. Reverse and forward primers were used for detecting the genes in study, as described by Rebelo and colleagues (2018) and Borowiak and colleagues (2020). (Borowiak et al., 2020; Rebelo et al., 2018)

The products of the PCR reaction were further analyzed by electrophoresis, through an agarose gel at 1,5% weight-volume (Agarose Ultrapure grade, NZYTech), placed in 1x TBE buffer, stained with GreenSafe at a concentration of 1% (GreenSafe Premium, NZYTech). A molecular ladder and positive controls for the targeted *mcr* genes were loaded onto the gel along with the isolates, allowing comparison of the molecular weight of the DNA fragments obtained. A negative control was used as well. Electrophoresis was run on the 1,5% agarose gel containing the PCR products, the molecular ladder, and the controls, for 35 minutes at 90V. Results were read through an UV transilluminator (BioRad, California, USA), and images were processed and edited using ChemiDoc software (BioRad).

2. Results

2.1. Characterization of colistin resistant isolates

Three *E. coli* isolates (H1/S300, H12/S300 and H23/300) displayed a MIC of 4 µg/mL or higher, resulting in a total prevalence of colistin-resistance of 6%. All of these isolates belonged to group H (68% of the total of samples), whereas no colistin-resistant isolates were recovered from group S. One isolate (H16/Cb300), despite presenting growth in a selective medium with colistin, displayed a MIC lower than 1 µg/mL, therefore, it cannot be considered resistant to colistin.

Isolates H1/S300 and H12/S300 were recovered from post-enrichment SCAi + Col, while isolate H23/300 was recovered from direct TBX + Col and the isolate H16/Cb300 was recovered from TBX medium after incubation on Brilliant Green Bile Broth, displaying the typical *E. coli* blue iridescent colonies (Table 1).

Out of the 50 samples tested, 22 (44%) displayed growth on TBX + Col, SCAi + Col or on simple TBX medium after incubation on Brilliant Green Bile Broth. However, after the subsequent biochemical testing and assessment of the typical appearance in

the media used, only four isolates were identified as *E. coli* (8%). No *Klebsiella* spp. isolates were recovered.

The resistant isolates were obtained from animals between the ages of 2 months and 7 years, with 100% of the food provided consisting in dry pellets, store bought and with no history of recent antibiotic administration. Only one of these animals had access to the outside. No *mcr* genes (1 – 9) were detected in any of the isolates tested.

Interestingly, all three colistin-resistant strains showed no resistance to other antibiotics tested.

Table 1. Characterization of colistin-resistant isolates obtained from companion animals – Species identification, MIC_{Col}, presence of *mcr* genes, animal identification, age, recent antimicrobial treatment, diet, outside access and history of gastrointestinal symptoms.

Isolate characteristics					Animal characteristics					
Isolate ID	Bacterial species	Culture medium	MIC _{Col} (µg/ml)	<i>mcr</i>	Species	Age	AMTx	Diet	Outside access	G.I. symptoms
H1/S300	<i>E. coli</i>	SCAi + Col	> 16	No	Cat	4y	No	Pellet	Yes	No
H12/S300	<i>E. coli</i>	SCAi + Col	4	No	Dog	2 mo	No	Pellet	No	Yes
H23/300	<i>E. coli</i>	TBX + Col	16	No	Cat	7y	No	Pellet	No	No

Antimicrobial susceptibility testing on isolates not resistant to colistin

A total of 37 *E. coli* isolates were detected, with 14 isolates obtained from group S (37,8%) and 23 isolates from group H (62,2%); In five cases, two different morphotypes originated from the same sample. The *E. coli* isolates recovered presented mainly resistance to ampicillin (32,4%), tetracycline (32,4%), doxycycline (21,6%), sulfamethoxazole-trimethoprim (10,8%), cefazolin (8,1%) and nitrofurantoin (5,4%). Comparing both groups, the isolates from group S present resistance to ampicillin (57,1%), tetracycline (57,1%), doxycycline (42,9%), sulfamethoxazole-trimethoprim (21,4%), cefazolin (14,3%), and streptomycin (7,1%) (Figure 2), while isolates from group H displayed resistance against tetracycline (21,7%), ampicillin (13,0%), doxycycline (8,7%), nitrofurantoin (4,3%), sulfamethoxazole-trimethoprim (4,3%) and cefazolin (4,3%); no resistance to streptomycin was detected (Figure 2). One single ESBL-producing *E. coli* strain was detected within this group in a 14-year-old dog, with no recent history of antimicrobial treatment and no G.I. symptoms associated (Figure 3).

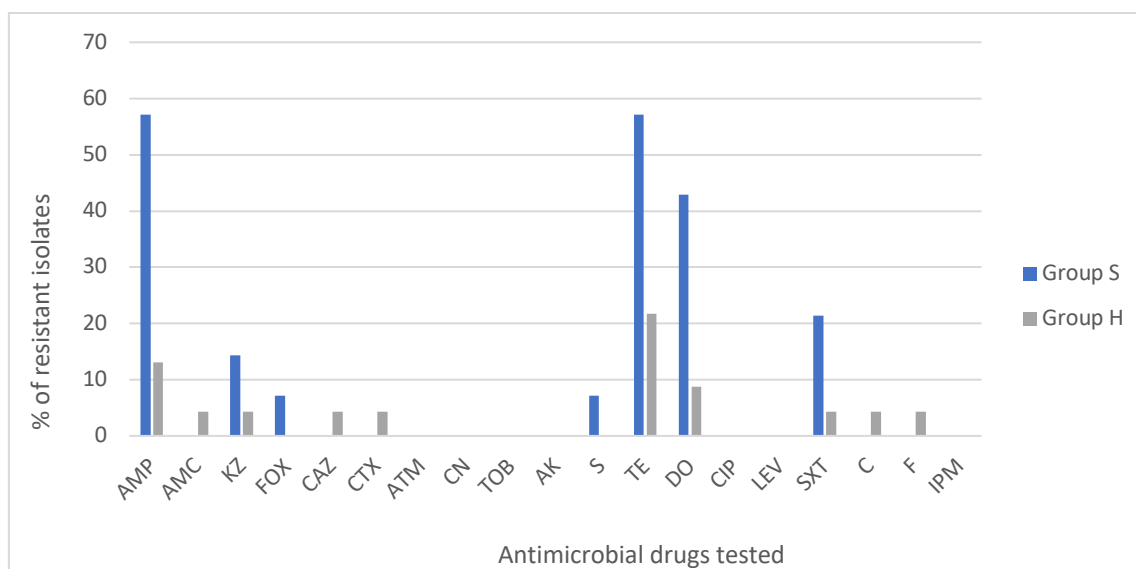


Figure 2. Percentage of antibiotic resistance detected among *E. coli* isolates (n=37) recovered from companion animals housed in a shelter (S; n=16) and attended at UPVet (H; n=34). [AMP – Ampicillin; AMC – Amoxicillin – Clavulanate; KZ – Cefazolin; FOX – Cefoxitin; CAZ – Ceftazidime; CTX – Cefotaxime; ATM – Aztreonam; CN – Gentamycin; TOB – Tobramycin; AK – Amikacin; S – Streptomycin; TE – Tetracycline; DO – Doxycycline; CIP – Ciprofloxacin; LEV – Levofloxacin; SXT – Sulfamethoxazole – Trimethoprim; C – Chloramphenicol; F – Nitrofurantoin; IPM – Imipenem]

Four of the 37 isolates obtained (10,8%) were multidrug resistant (MDR), with group S presenting three MDR isolates (21,4%) and group H one MDR isolate (4,3%).



Figure 3. Antimicrobial susceptibility testing for confirmation of ESBL - producing *E. coli* on isolate H16/Cb300.

3. Discussion

The generally available information regarding colistin-resistance in companion animals is scarce, as most studies focus on food-producing animals; however, interesting reports found a prevalence of colistin-resistant Enterobacteriaceae ranging

between 0,7% and 14% (Hamame *et al.*, 2022; Joosten *et al.*, 2020; Lei Lei *et al.*, 2017; Pomba, 2020; Rumi *et al.*, 2019; Zhang *et al.*, 2016). Therefore, the 6% prevalence found in this study corroborates the results of the existent data, although the comparison should be made with caution, considering that previous research was performed for longer periods of time, on larger and slightly different populations and, finally, concerning different biological materials, such as urine samples or skin tissue.

In the present work, no colistin-resistant strain was recovered from group S. It is, nonetheless, important to consider that there is a difference between the number of samples obtained for each of the groups (*i.e.*, n=16 for group S vs. n=34 for group H). Future studies, comprising a larger number of samples originating in sheltered animals, will allow a more accurate comparison between groups.

All colistin resistant isolates in the present study were identified as *E. coli*, while no *Klebsiella* spp. isolates were obtained. A number of studies aiming to detect colistin resistance in Enterobacteriaceae have recovered both of these bacteria from companion animals, with *E. coli* being more frequently isolated than *Klebsiella* spp. (Hamame *et al.*, 2022; Lei Lei *et al.*, 2017; Rumi *et al.*, 2019). These studies, however, were able to obtain a greater number of isolates, which can account for the greater prevalence of *Klebsiella* spp. In addition, only SCAi + Col medium was used in the present study; therefore, non-colistin resistant *Klebsiella* spp. could not have been detected in this way.

In what concerns the animals from which colistin-resistant strains were isolated, history of recent antibiotic treatment does not seem to play a pivotal role in the isolation of colistin resistant bacteria (Table 1). In fact, none of the animals from which these isolates originated had a history of recent antimicrobial drug intake, whereas in samples originating from animals with a history of recent antibiotic administration, no colistin resistant isolates were obtained.

Access to the outside does not seem to be a relevant risk factor either, as colistin-resistant strains were found in both animals with and without access to the outside. Moreover, no colistin-resistant *E. coli* was found in sheltered animals, suggesting that close cohabitation with humans might be more important than exposure to environmental sources (*e.g.*, water, contacts with wild birds).

Concerning animals from group H, the *E. coli* colistin-resistant isolates were obtained from animals eating store bought dry pellets, not being recovered from animals with home-made feeding diets. The role of commercial feeding on the faecal colonization with colistin-resistant isolates is still being discussed; however, previous studies have suggested that an association between commercial pet food (in particular, poultry-based) and colistin-resistant bacteria may exist (Lei Lei *et al.*, 2017).

Gastrointestinal (G.I.) tract disease does not seem to have an influence on isolation of colistin resistant *E. coli*, given the fact that, within the group of resistant isolates, only one of the animals showed symptoms of G.I. disease (diarrhea), while the remaining animals were presented to consultation for unrelated reasons. Future studies on the subject, including a similar gathering of information related to the animals' lifestyle would require a higher number of samples, so risk factors for colistin resistance can be thoroughly identified.

No *mcr* genes (1 – 9) were detected in these isolates, which can be explained either by the presence of colistin resistance mediated by chromosomal mechanisms, or a new variety of *mcr* that cannot be detected by the primers used in this study. Further genomic characterization, such as Whole Genome Sequence, would be required, so to understand the exact mechanism behind these resistances. Previously published studies, performed on companion animals, have been able to recover *mcr* genes; nonetheless, a proportion of resistances due to chromosomal mechanisms has also been reported (Hamame *et al.*, 2022).

Regarding antimicrobial susceptibility testing, the colistin-resistant strains were susceptible to every other antimicrobial drug tested. Other studies, although not performed in companion animals, suggest that *mcr*-carrying plasmids may encode resistance genes for other classes of antimicrobials (Li *et al.*, 2018), but no *mcr* genes are present in the isolates obtained in this study.

In this study, 37 *E. coli* isolates, not displaying resistance to colistin, were isolated in both groups; resistance against tetracycline, ampicillin, doxycycline, and sulfamethoxazole-trimethoprim were found frequently. Overall, it was possible to conclude that group S, originating from sheltered animals, displays a greater percentage of resistant isolates. However, group H isolates displayed resistance against a greater number of antimicrobial drugs (i.e., chloramphenicol, nitrofurantoin, ceftazidime, and cefotaxime) which are not present in group S. The presence of an ESBL-producing *E. coli* in group H accounts for resistance to both ceftazidime and cefotaxime. The presence of a greater proportion of resistant isolates, as well as a greater proportion of MDR isolates, in group S was most likely a result of a higher exposure to environmental sources (e.g. water, contacts with wild birds). Despite being hard to obtain a well-documented previous history of these animals, no antimicrobial treatment was recorded in sheltered animals.

The fact that the diversity of resistances is greater in group H may be explained not only by the fact that this group comprises almost twice the number of samples, but also by the fact that pets, living in close contact with humans, may be exposed, in different ways, to a greater number of antimicrobial substances or resistant organisms.

A study from Joosten and colleagues (2020), performed in companion animals across three European countries points to ampicillin, sulfamethoxazole and tetracycline as the most common resistances found in these animals. In the present study, ampicillin, tetracycline, and doxycycline were the most common resistances found, followed by sulfamethoxazole – trimethoprim. Therefore, both data consider the same antimicrobials as the most frequently obtained resistances. A study from Marchetti et. al (2021), focused on stray dogs and pet dogs, concluded that the most commonly found resistances were to ampicillin, tetracycline, and streptomycin, with the greater proportion of resistant isolates found in the stray dog group, which also reveals similar findings to those of the present study. However, the researchers found a greater number of ESBL - producing *E. coli* isolates. Regarding this work, in all animals undergoing or with recent history of antibiotic treatment, the most commonly administered antibiotic was amoxicillin – clavulanate; this does not agree with the low proportion of amoxicillin – clavulanate resistant isolates found, so further research may come to establish a relevant association between the β -lactam (amoxicillin and ampicillin) antimicrobials (Joosten *et al.*, 2020; Marchetti *et al.*, 2021).

Contrary to expectation, in what concerns the recovery of colistin resistant isolates, most *E. coli* isolates (two out of three) were recovered from SCAi + Col medium, despite the fact that a TBX + Col medium was used for the detection of these strains. One of these isolates, recovered from SCAi + Col, did not display the typical dome-shaped, aqueous appearance that has been previously described (Van Kregten *et al.*, 1984) consisting, instead, of small orange – yellow colonies; while this particular strain was able to grow on this medium, containing colistin, no growth was observed when the isolate was plated in simple SCAi medium. An essay involving the use of colistin, and myo-inositol was performed, revealing that myo-inositol does not seem to alter the growth of these strains. So, it was not possible to conclude what substance is influencing it and therefore, further genomic, and biochemical research would be required to draw a relevant conclusion.

An ESBL-producing *E. coli* was recovered from an asymptomatic 14-year-old dog, with no recent history of antibiotic treatment. Geriatric patients have often been exposed, throughout their lives, to a greater number of antimicrobial substances and may have present conditions which require treatment with such drugs, which can account for a greater diversity of resistances (Marchetti *et al.*, 2021). Nonetheless, this is, *per se*, a worrisome finding. ESBL-producing strains display resistance to β -lactam antibiotics, commonly used for treatment of *E. coli* infections. These mainly plasmid-mediated mechanisms have been reported to encode resistance genes to other classes of antimicrobials, such as aminoglycosides and fluoroquinolones, also of great

importance. Moreover, presence of this plasmid may facilitate the spreading and acquisition of these resistances by other bacteria (Huang *et al.*, 2020). ESBL-producing strains tend to exhibit a multi-drug-resistant AMR profile, which can be observed in the isolate obtained in this study (Huang *et al.*, 2020). Therefore, the number of resistances comprised in these isolates further threatens the reliability of several antimicrobial substances and thus evidences their importance to animal and human health.

The proximity between pets and humans increases the risk these animals pose for human health, facilitating the zoonotic transmission of bacteria (Overgaauw *et al.*, 2020). The presence of MDR bacteria and an ESBL-producing strain among these animals, even if in low numbers, is worrisome. In addition to living in close contact with humans, as well as other animals (oftentimes, more than one species), and being increasingly included in the routine of the household (*e.g.* vacations or events that require moving to a different location), these animals will circulate through a variety of environments and contact with a variety of potential hosts, further increasing the possibility of spreading bacteria with such harmful characteristics. The same is true and even more concerning, in what relates to stray animals. These are animals which can possibly roam freely before being sheltered or rehoused and frequently with no access to adequate veterinary care. Therefore, presence and dissemination of MDR bacteria is facilitated in this group. This impacts not only animal health and possibly environmental health, but also, and inevitably, human health and eventually, the reliability of antimicrobial drugs as efficient treatments for known diseases.

It is crucial that data on the prevalence of colistin-resistant bacteria and also MDR isolates, is continuously investigated, updated, and monitored, in as many locations and different populations as possible. While a relevant association between the animals' living conditions and the characteristics of the isolates obtained could not be established in the present study, these findings further highlight the pivotal role of continued antibiotic stewardship, and increased awareness and knowledge-seeking on the matter, both from owners and veterinary professionals.

Chapter Two

Colistin-Resistant *E. coli* and *Klebsiella* spp. in calves

1. Introduction

An ever-growing human population will, inevitably, lead to a greater number of challenges to ensure that people will have access to safe, nutritious, and healthy food. Dairy farming is essential to food supply and the number of dairy cows, as well as milk production per cow, has grown continuously (Garcia *et al.*, 2019). Likewise, veterinary care and the need for antibiotics have been increasing. Their use in animal husbandry and veterinary medicine has resulted in healthier and more productive farm animals, ensuring the welfare and health of both animals and humans.

However, as in human medicine, the use of antimicrobial drugs in veterinary medicine creates a selective pressure for the emergence of antimicrobial-resistant bacteria, including animal pathogens, human pathogens that have animal reservoirs, and commensal bacteria that are present in animals. Drug-resistant bacteria selected by this selective pressure can spread to humans either by the food supply (e.g. meat, fish, eggs and dairy products), direct contact with animals or, more indirectly, through environmental pathways (Valiakos & Kapna, 2021).

Dairy cows are frequently medicated with antimicrobial drugs for the treatment of mastitis, which is a common condition among these animals; while milk from treated animals cannot be made available for human consumption, it can be discarded through watercourses, manure, or more commonly, fed to calves (Firth *et al.*, 2021). The presence of small amounts of antimicrobial residues in this milk may pressure the enrichment of antibiotic-resistant bacteria and, even more worryingly, may promote the acquisition of resistance genes from sensitive bacteria (EFSA, 2017). *E. coli* and *Klebsiella pneumoniae*, belonging to the Enterobacteriaceae family, are two of the most common Gram-negative pathogens found in dairy farms, frequently causing mastitis (Tartor *et al.*, 2021). These bacteria can be found in the normal gut flora of animals and humans and have pathogenic potential (Bista *et al.*, 2020; Harada *et al.*, 2016).

Since the discovery of colistin, there has been a widespread use of this antimicrobial drug in veterinary medicine (Kempf *et al.*, 2016; Kieffer *et al.*, 2014; Nation & Li, 2009). While countries, such as China, have banned the use of colistin in the veterinary practice, colistin usage within the European Union (EU) is still possible, and several formulations are still available for food-producing animals (Kumar *et al.*, 2020). In 2012, despite the differences across countries, the usage of colistin in these groups of animals across the EU was 600 times higher than the usage in human medicine

(Binsker *et al.*, 2022). The most frequent administrations of colistin occur in swine and poultry (Binsker *et al.*, 2022; Kempf *et al.*, 2016).

Although the administration of colistin in cattle is sporadic, it is mainly intended for the treatment of diarrheal conditions in veal calves. Nonetheless, as previously reviewed by Valiakos & Kapna (2021), resistance to colistin has been detected within cattle, mainly on *E. coli* isolates harboring *mcr-1* and *mcr-2* genes; however, varieties from 3 to 10 have also been reported in these animals. While *E. coli* is considered to be the main host of *mcr-1* genes, the *mcr* variants 1, 2, 3, 7, 8 and 9 have been recovered from several *Klebsiella* spp. strains, originating from both animal and human sources (Chandler *et al.*, 2020; Luo *et al.*, 2020).

The global dissemination of mobile colistin resistance genes (*mcr*) in animals, humans, and the food chain, pose a threat to human, animal, and environmental health, which must be continuously monitored and studied.

1.1. Aim of the study

The aim of the study was to evaluate the presence of colistin-resistant *E. coli* and *Klebsiella* spp. (i) on bovine milk samples obtained from cows being treated for mastitis, and (ii) on fecal samples from calves fed with the same milk. The phenotypical characterization of bacterial isolates, including antimicrobial susceptibility testing was also performed.

1.2. Materials and Methods

1.2.1. Sampling

For this study, thirteen dairy farms were sampled, and two types of samples were collected from each farm: 13 samples of pooled raw milk from antibiotic treated cows (group VL) and 13 samples of pooled fecal samples from calves that ingested raw milk from antibiotic treated cows (group VF). A questionnaire was provided to the farm owners, regarding the recent antibiotic administration to the animals in study. This study had the approval of the Organismo Responsável pelo Bem-estar Animal do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto (ORBEA ICBAS-UP).

1.2.2. Culture and isolation of *E. coli* and *Klebsiella* spp.

Immediately after reception, samples were left for one hour at room temperature. After this period, 100 µL of this suspension were spread in selective media – TBX, TBX + 3,5 mg/L of colistin, SCAi + 3,5 mg/L of colistin, following the protocol and incubation

times described in Chapter One (page 4). In contrast to the previously described, no processing of post enrichment was performed at 18h of incubation.

1.2.3. Identification of isolates

Colonies growing on TBX, TBX + Col or SCAi + Col were observed for typical *E. coli* or *Klebsiella* spp. appearance. Further biochemical tests, result interpretation, isolates' DNA extraction and respective storage were performed as described in Chapter One (page 5).

1.2.4. Antimicrobial susceptibility testing

Antimicrobial susceptibility assessment was performed on all *E. coli* and *Klebsiella* spp. isolates obtained from TBX, TBX + Col or SCAi + Col. The selection of colonies, antimicrobial susceptibility testing method and antimicrobial drugs tested were previously described in Chapter One (page 5).

1.2.5. Colistin resistance – Broth Microdilution Method

The microbroth dilution method using the 96-microtiter plate was employed, following the guidelines proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute, 2018; European Committee on antimicrobial susceptibility testing - EUCAST, 2020). This protocol was thoroughly described in Chapter One (page 6).

1.2.6. PCR for detection of *mcr* genes

The detection of *mcr* genes was performed on all isolates recovered from selective media with colistin, using a Multiplex Polymerase Chain Reaction (PCR), targeting *mcr* 1 to 5 (Rebelo *et al.*, 2018), and *mcr* 6 to 9 (Borowiak *et al.*, 2020). The processing of PCR product and reading of results is described in detail in Chapter One (page 7).

2. Results

2.1. Characterization of colistin resistant isolates

From a total of 15 isolates obtained from TBX + Col medium, only one (6%) had a MIC_{Col} higher than 16 µg/mL (therefore being considered resistant to colistin), while two isolates displayed intermediate resistance to colistin (MIC_{Col} of 2 µg/mL). The

colistin-resistant strain was recovered from a milk sample (group VL) of cows undergoing antibiotic treatment, whereas the isolates with intermediate resistance were recovered from fecal samples (group VF). No other colistin-resistant isolate was recovered, neither from group VF nor group VL.

Fourteen *E. coli* isolates were recovered from four fecal samples (30%) plated in TBX + Col medium. Regarding milk samples, only one (7%) *E. coli* isolate was detected on the same culture media. *Klebsiella* spp. was not detected, since growth on SCAi + Col was not observed in any of the cultured samples. No *mcr* genes (1 – 9) were detected in these isolates (Table 2).

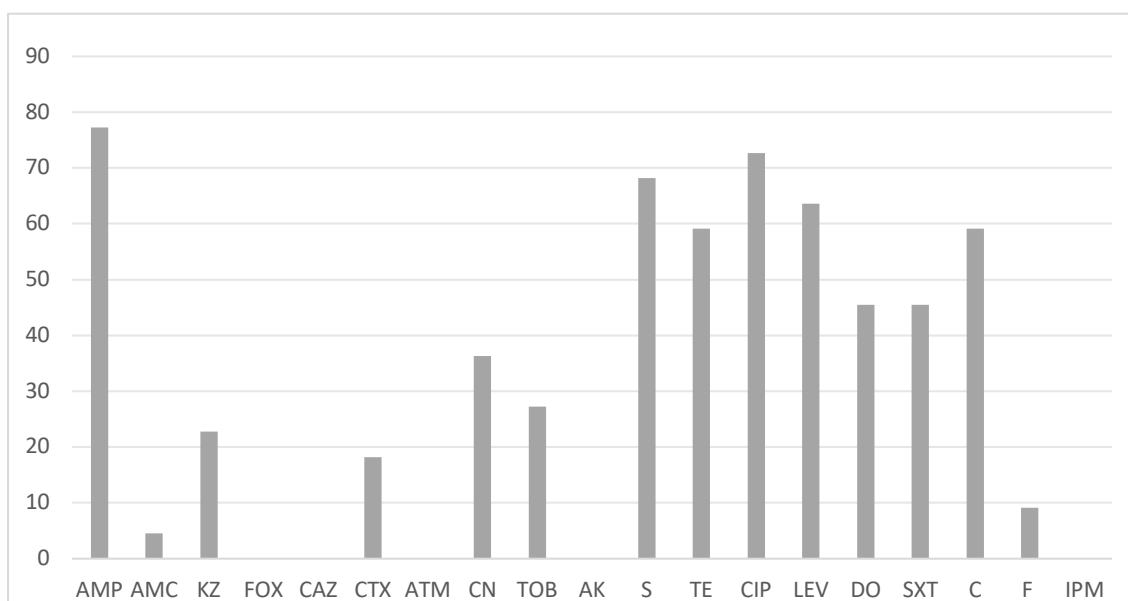
Table 2. Characterization of *E. coli* isolates obtained from raw milk collected from antibiotic treated cows (group VL) fecal samples from calves (group VF) – sample, culture media, isolate ID, MIC_{Col}, presence of *mcr* 1 – 9, and antimicrobial resistance profile. [AMP – Ampicillin; AMC – Amoxicillin – Clavulanate; KZ – Cefazolin; FOX – Cefoxitin; CAZ – Ceftazidime; CTX – Cefotaxime; ATM – Aztreonam; CN – Gentamycin; TOB – Tobramycin; AK – Amikacin; S – Streptomycin; TE – Tetracycline; CIP – Ciprofloxacin; LEV – Levofloxacin; DO – Doxycycline; SXT – Sulfamethoxazole – Trimethoprim; C – Chloramphenicol; F – Nitrofurantoin]

Origin	Sample	Culture medium	ID	MIC _{Col}	<i>mcr</i> 1 - 9	AMR profile
VF	1	TBX + Col	300	< 1 µg/mL	No	AMP, S, TE, DO, CIP, LEV, SXT
VF	1	TBX + Col	301	2 µg/mL	No	AMP, ATM, CN, S, TE, DO, CIP, LEV, SXT, C
VF	1	TBX + Col	302	2 µg/mL	No	CIP, LEV, C
VF	1	TBX + Col	303	< 1 µg/mL	No	CIP, LEV, C
VF	1	TBX + Col	304	< 1 µg/mL	No	AMP, C
VF	1	TBX + Col	305	< 1 µg/mL	No	CIP, LEV, C
VF	1	TBX + Col	306	< 1 µg/mL	No	AMP, S, TE, DO, CIP, LEV
VF	1	TBX + Col	307	< 1 µg/mL	No	AMP, CN, S, CIP, LEV, SXT, C
VF	1	TBX + Col	308	< 1 µg/mL	No	AMP, S, TE, DO, CIP, LEV
VF	1	TBX + Col	309	< 1 µg/mL	No	AMP, S, TE, DO, CIP, LEV
VF	3	TBX + Col	301	< 1 µg/mL	No	AMP, S, SXT
VF	4	TBX + Col	300	< 1 µg/mL	No	AMP, S, TE, DO, CIP, LEV, SXT, C
VF	9	TBX + Col	300	< 1 µg/mL	No	AMP, S, TE DO, CIP, LEV, SXT, C
VF	9	TBX + Col	302	< 1 µg/mL	No	AMP, AMC, CN, TOB, S, DO, CIP, SXT, C, F
VL	12	TBX + Col	300	> 16µg/mL	No	AMP, TE, DO, C, F

Antimicrobial susceptibility testing was performed, in resistant and non-resistant isolates, allowing to conclude that the isolate displaying resistance to colistin was also resistant to ampicillin, tetracycline, doxycycline, chloramphenicol and nitrofurantoin, thus being considered a multidrug resistant isolate (Magiorakos *et al.*, 2012).

Isolates presenting a MIC_{Col} of 2 µg/mL were recovered from one fecal sample pool from calves. These animals were fed with milk originating from cows treated with amoxicillin and penicillin. Resistance against fluoroquinolones and chloramphenicol were observed to both isolates (Table 2). However, separately, isolates also showed resistance to streptomycin, tetracycline, doxycycline, and sulfamethoxazole–trimethoprim.

Figure 4. A total of 22 non-colistin resistant *E. coli* isolates was obtained from fecal samples (group VF). The most frequent resistances were against ampicillin (77,3%), ciprofloxacin (72,7%), streptomycin (68,2%), levofloxacin (63,6%), chloramphenicol (59,1%), tetracycline (59,1%), doxycycline (45,5%), sulfamethoxazole – trimethoprim (45,5%), gentamicin (36,4%) and tobramycin (27,3%) (Figure 3). Out of the total 22 isolates, 14 (64%) were multidrug resistant. Finally, but also noteworthy, five out of 22 isolates (22,7%) were considered ESBL-producing *E. coli*. These isolates were recovered in farms with a history of recent use of amoxicillin, penicillin, lincomycin, neomycin, ceftiofur and quinolones, such as enrofloxacin and levofloxacin.



3. Discussion

This research had a rather exploratory purpose, with a very small number of samples. Therefore, the isolation of a colistin-resistant strain in milk samples from cows undergoing antibiotic treatment should be highlighted, but any interpretation should be extremely conservative. The same is true for the fact that no colistin-resistant strain was isolated from faecal samples, although it has been shown that these strains can be transmitted through milk. While the majority of previous studies in cattle has been able to recover colistin resistant isolates and *mcr* genes from faecal samples, these have also been isolated from waste milk samples and from samples taken at slaughter (Valiakos & Kapna, 2021).

The recovery of a colistin resistant isolate from a farm where no colistin was being used could be explained by a co-selection mechanism, involving other AMR genes, present in the same plasmid, as suggested in previous studies (Cao *et al.*, 2020; Dominguez *et al.*, 2018). However, as no *mcr* genes were detected, further genomic characterization would be required to determine the exact mechanism for colistin resistance and emergence in this isolate. In a previous study involving cow's milk, Liu *et al.* (2020) reported a prevalence of colistin resistance of 2%, out of a total of 249 *E. coli* isolates obtained. In addition, Liu and colleagues found a correlation between colistin resistance and the presence of *mcr-1* gene, as well as ESBL-producing strains; neither of which were found in this research. Another study from Tartor *et al.* (2021) reported a 52,1% prevalence of colistin-resistant isolates in bovine milk, with a 77% prevalence of *mcr* genes. Apart from considering a larger number of samples, these authors consider a MIC_{Col} cut off for colistin resistance of 2 µg/mL or higher, which can also account for a higher number of isolates recovered. Similar prevalences may be obtained in future research if a higher number of sampled farms could be obtained.

While other authors have been able to recover *Klebsiella* spp. isolates from both milk and faecal samples (Nobrega *et al.*, 2021; Yang *et al.*, 2021), no isolate of this bacterial genera was obtained. Besides the fact that previous studies were performed on larger populations, it must be emphasized that only SCAi + Col medium was used in the present study, which precludes the detection of non-colistin resistant *Klebsiella* spp.

In this study, resistances against ampicillin, tetracyclines and fluoroquinolones were frequent, which is consistent with the frequent administration of β-lactam and fluoroquinolone drugs in the sampled farms. On the contrary, the great number of chloramphenicol-resistant isolates was not expected, considering that the administration of this drug is forbidden in food-producing animals in the EU since 1994 (Schwarz *et al.*, 2004). This suggests that a cross-resistance mechanism may be present, for example, with genes encoding resistance against both chloramphenicol and florfenicol, which has

been previously reported (Schwarz *et al.*, 2004). Obtaining a more detailed history of these farms in terms of respiratory disease would be important to confirm this hypothesis.

Several studies demonstrated the effect of waste milk in the excretion of AMR bacteria from calves; the most commonly reported resistances include fluoroquinolones and cephalosporins. It is also reported a higher proportion of β -lactam resistant isolates in calves fed with waste milk (Firth *et al.*, 2021). Therefore, it is possible to conclude that the data obtained in this study agrees to previously published information.

In summary, despite the fact that only one colistin-resistant *E. coli* was obtained in milk not intended for human consumption, this finding is worrisome. Firstly, because this competence (colistin resistance) may exist even in the absence of direct selective pressure; secondly, because this strain is being transmitted to other food-producing animals (after rearing calves will be slaughtered for meat production) and finally, because of the possibility that this strain (and others that may exist) may spread in the environment and thus colonize other animals and biotopes, perpetuating its existence (Garcia *et al.*, 2019; Liu *et al.*, 2020)

The findings in the present study highlight the importance of a strict enforcement of laws regarding antimicrobial use in food-producing animals. Moreover, it also evidences the pivotal role of veterinary professionals in responsible decision-making processes, in what concerns the circumstances of antimicrobial administration. Therefore, continued investigation on the matter is critical, further raising awareness and increasing knowledge to all parts involved – veterinarians, owners, and producers.

Chapter Three

Detection of *Campylobacter* spp. in companion animals

1. Introduction

Campylobacteriosis in Europe has been the most commonly reported zoonotic disease in humans since 2005 and the number of cases across the continent has increased (Iannino *et al.*, 2019). *Campylobacter* infection can cause severe, life-threatening disease resulting in death, particularly in young children or immunocompromised patients. Complications are possible and include reactive arthritis and Guillain-Barré syndrome (Iannino *et al.*, 2019; World Health Organization, 2020).

These helical-shaped Gram-negative bacteria also possess the ability to colonize asymptomatically the intestinal tract of various species (World Organisation for Animal Health (OIE), 2017). *Campylobacter* spp. shows optimal growth in environments with a low oxygen tension (Facciola *et al.*, 2017; Pendleton *et al.*, 2015); however, its genomic flexibility allows it to adapt to a variety of hosts and environments (Facciola *et al.*, 2017; Lemos *et al.*, 2021; Mughini-Gras *et al.*, 2021; Pendleton *et al.*, 2015; World Health Organization, 2020).

All *Campylobacter* spp. are potentially pathogenic; however, *C. jejuni* is most frequently associated with human infection, followed by *C. coli* and *C. lari*, while the role of *C. upsaliensis* as a human pathogen is still being discussed (Leonard *et al.*, 2011; Wieland *et al.*, 2005). *C. jejuni* and *C. upsaliensis* are the most commonly isolated species in companion animals (Acke, 2018). In the majority of the cases where these species are isolated, animals are sub-clinically infected and remain asymptomatic. However, younger, or immunocompromised animals are prone to develop clinical signs of *Campylobacteriosis*. These signs include a mild to moderate diarrhea (which is generally self-limiting and does not require antibiotic treatment), fever, and stomach cramps. Nausea and vomiting may accompany the diarrhea. Sometimes, *Campylobacter* infections are associated with irritable bowel syndrome, temporary paralysis, and arthritis. *Campylobacter* can occasionally cause life-threatening infections in immunocompromised people (Acke, 2018; Iannino *et al.*, 2019).

A previous study has established an association between infection with *Campylobacter* spp. (*C. jejuni*, in particular) and two cases of acute and progressive polyradiculoneuritis, in which the patients displayed symptoms, comparable to those of Guillain-Barré syndrome in humans (Kim *et al.*, 2021).

The sharing of a living space with a dog has been described as a risk factor for *Campylobacteriosis*, with 9 to 25% of human *Campylobacteriosis* cases being

associated with companion animals across Europe (Gras *et al.*, 2013). Considering the settings in which most pets live and the proximity they share with humans and other animals, the presence of *Campylobacter* spp. in companion animals poses a threat to public health, not only considering the possibility of pet-to-human transmission (regarding particularly *C. jejuni*) (Damborg *et al.*, 2004), but also considering the rise of MDR bacteria, the circulation of these organisms between humans and their potential for spreading through several locations and diverse hosts (Iannino *et al.*, 2019).

1.1. Aim of the study

This work aims to give an insight into the presence of *Campylobacter* spp. in different populations of dogs and cats and characterize the obtained isolates in terms of antimicrobial resistance profiles.

1.2. Materials and Methods

1.2.1. Sampling

For this preliminary assessment, between December 27th, 2021, and February 14th, 2022, a total of 50 rectal swabs was collected: 16 were obtained from dogs (n = 12) and cats (n = 4) housed in a shelter, located in Coimbra, Portugal (group S) and 34 rectal swabs were obtained from dogs (n = 24) and cats (n = 10) presented to consultation at the Veterinary Hospital of the University of Porto (UP Vet) (group H). For both groups in study, a written consent was provided to the persons in charge and information was gathered on the animals' general living conditions, dietary habits, and recent history of antimicrobial treatment (Annex A and B). This study had the approval of the Organismo Responsável pelo Bem-estar Animal do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto (ORBEA ICBAS-UP).

1.2.2. Culture and isolation of *Campylobacter* spp. from rectal swabs

Rectal swabs were added to 10 mL of Bolton broth (Bolton Broth, Oxoid + 5% horse blood lysed, Oxoid) and incubated for 48h at 41,5°C under microaerophilic conditions generated by CampyGen 2,5 mL (ThermoScientific). Then, 100 µL of post-enrichment were placed in Blood Agar (BA) (Biokar Diagnostics + 5% Horse Lysed blood, Oxoid) through a 0,65 µM filter and then incubated for 48h at 41,5°C, under microaerophilic conditions, according to ISO 11290 (ISO, 2017). Colonies identified as *Campylobacter* spp. were subcultured in BA and incubate at 41,5°C for 48h, under

microaerophilic conditions. A duplicate culture in BA was also performed and incubated at 41,5°C for 48h, in aerophilic conditions.

1.2.3. Identification of isolates

After the initial 48h incubation period, colonies were evaluated on BA. Colonies presenting typical *Campylobacter* spp. appearance were tested for oxidase (Oxidase Test Stick, Liofilchem) and oxidase-positive isolates were then observed through phase-contrast microscopy, for detection of typical morphology and motility. When the typical characteristics were observed, subculture in BA was performed (48h at 41,5°C, under microaerophilic conditions). Growth was then evaluated; if observed only on the BA incubated under microaerophilic conditions, DNA extraction was performed through the same protocol previously described for Enterobacteriaceae and the isolates were stored in 1 mL of Brain Heart Infusion (BHI) (Sigma – Aldrich), at -80°C.



Figure 5 - Example of bacterial colonies displaying typical *Campylobacter* spp. appearance in Blood Agar.

1.2.4. Antimicrobial Susceptibility Testing

Susceptibility to antimicrobial agents was tested using the disk diffusion method according to the Kirby-Bauer method, using the following antimicrobial drugs (Oxoid): Ampicillin (AMP) (10 µg), Amoxicillin-Clavulanate (AMC) (30 µg), Tetracycline (TE) (30 µg), Imipenem (IPM) (10 µg), Ciprofloxacin (CIP) (5 µg), Gentamycin (CN) (10 µg) and Erythromycin (E) (15 µg). Results were interpreted following the Clinical Laboratory Standards Institute (CLSI) guidelines, and isolates were classified as Susceptible, Intermediate or Resistant (CLSI, 2015).

1.2.5.PCR Multiplex for confirmation of *Campylobacter* spp. presence

A multiplex Polymerase Chain Reaction (PCR) technique was performed to confirm *Campylobacter* Genus and for simultaneous identification to the species level. Primers selected and conditions of the assay were according to the protocol described by Yamazaki-Matsune and colleagues (2007). Controls targeting *Campylobacter* spp., *C. jejuni*, *C. fetus*, *C. lari*, *C. upsaliensis*, *C. coli* and *C. hyointestinalis* were included. The products of this reaction were analyzed by electrophoresis, through a 1,5% (weight-volume) agarose gel (Agarose Ultrapure grade, NZYTech), in 1x TBE buffer stained with 1% GreenSafe (GreenSafe Premium, NZYTech). Results were read through an UV transilluminator (BioRad GelDoc XR) and images were processed and edited using ChemiDoc software (BioRad).

2. Results

Out of the 50 rectal swabs analyzed, a total of 15 (30%) displayed typical *Campylobacter* spp. colonies. However, after phenotypical and genotypical assessment, only five of these initial 50 samples (10%) were confirmed as *Campylobacter* spp. Overall, six *Campylobacter* spp. isolates were recovered, three belonging to group H (50%) and three belonging to group S (50%). Isolates from group H, from two dogs and one cat, were identified as *Campylobacter upsaliensis*, while isolates from group S, from two dogs, were identified as *Campylobacter jejuni*. Isolates were recovered from five adult animals and only one of these animals displayed chronic gastrointestinal symptoms; interestingly it was the only animal that have access to the outside.

None of these animals had a history of recent antibiotic treatment, and none included raw meat in their diet (Table 3). In one case, the owner reported recent gastrointestinal symptoms within the household, but further details were not provided. Immunocompromised individuals were not present in any of the households.

Regarding the antimicrobial susceptibility testing, three isolates were resistant to amoxicillin-clavulanate (50%), while two isolates were resistant to ampicillin (33,3%), two isolates were resistant to tetracycline (33,3%), and one displayed intermediate resistance to erythromycin. If the species is considered while analyzing these antimicrobial resistance results, it can be concluded that while the *C. jejuni* isolates were mainly resistant to amoxicillin-clavulanate (33,3%) and tetracycline (66,7%), with one intermediate resistance to erythromycin being included here. On the other hand, *C. upsaliensis* mainly displayed resistance to ampicillin (66,7%) and amoxicillin-clavulanate (66,7%).

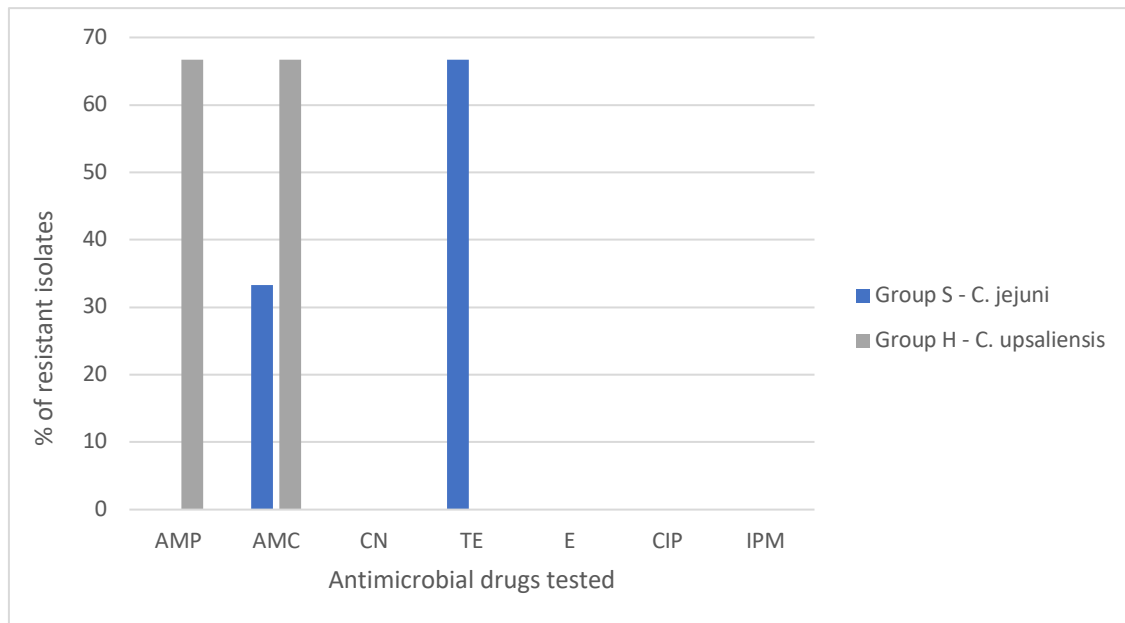


Figure 5. Percentage of resistance in *Campylobacter* spp. isolated from rectal swabs collected from dogs (n = 12) and cats (n = 4) housed in a shelter (group S) and from dogs (n = 24) and cats (n = 10) presented to consultation at UPVet) (group H). [AMP – Ampicillin; AMC – Amoxicillin – Clavulanate; CN – Gentamycin; TE – Tetracycline; E – Erythromycin; CIP – Ciprofloxacin; IPM – Imipenem]

Table 3. Characterization and origin of *Campylobacter* isolates obtained from rectal swabs collected from dogs (n = 12) and cats (n = 4) housed in a shelter (group K) and from dogs (n = 24) and cats (n = 10) presented to consultation at UPVet) (group H). [Isolate, species identification, antimicrobial resistance profile, animal and group of origin, raw meat intake in diet, antimicrobial treatment, access to the outside, gastrointestinal symptoms, and presence of vulnerable individuals (e.g., immunocompromised, pregnant, etc.) and gastrointestinal symptoms within the household). [AMC – Amoxicillin – Clavulanate; AMP – Ampicillin; TE – Tetracycline]

Isolate			Animal						Household	
ID	Species	AMR	Animal	Group	Raw meat	A.M. Treatment	Outside access	G.I. symptoms	Vulnerable individuals	G.I. symptoms
CCO 11/2	<i>C. jejuni</i>	-	Dog	S	No	No	No	No	None	No
CCO 15/1	<i>C. jejuni</i>	TE	Dog	S	No	No	No	No	None	No
CCO 15/2	<i>C. jejuni</i>	TE, AMC	Dog	S	No	No	No	No	None	No
H2/1	<i>C. upsaliensis</i>	-	Cat	H	No	No	Yes	Yes	None	No
H19	<i>C. upsaliensis</i>	AMP, AMC	Dog	H	No	No	No	No	No	Yes
H25	<i>C. upsaliensis</i>	AMP, AMC	Dog	H	No	No	No	No	No	No

3. Discussion

Overall, *C. jejuni* was detected in 4% of the sampled animals, whereas 6% harbored *C. upsaliensis*. In a previous study, Lemos *et al.* (2021), found a prevalence of 14,44% and 3,2% *C. jejuni* and *C. upsaliensis* in dogs, respectively. Nonetheless, the prevalence obtained in this study, for both species, seems to be lower than previously reported by Leonard *et al.*, (2011) and Wieland *et al.*, (2005).

Among cats sampled in this study (n = 14), only one *C. upsaliensis* was obtained. Cats have been most frequently described to carry *C. helveticus*; however, this bacterial species is particularly difficult to distinguish from *C. upsaliensis* with the tests commonly used (Wieland *et al.*, 2005). Previously, Wieland *et al.*, (2005) reported the isolation of *C. upsaliensis* from cats.

Considering that pets live in much closer proximity to humans, and that *C. jejuni* is a pathogen common to both animals and humans, perhaps a greater prevalence of this bacteria was to be expected in the housed animals group. This, however, could not be verified in the present study, and further research is important in continuing to understand the role of pets as vectors of *C. jejuni*.

Despite efforts to conduct a rigorous and systematic questionnaire, it must be acknowledged that collecting accurate information is quite difficult, especially in what concerns sheltered animals. Therefore, it is likely that these dogs have had, in the past, different dietary regimens and different living conditions, which have potentially exposed them to different bacteria and, eventually, different antimicrobial drugs. It was not possible to ascertain this information in this study; research on larger populations and a more detailed history would be important, to obtain more accurate comparisons between these animals and house pets.

None of the animals in study had a history of recent antimicrobial treatment, nor did any of the diets include raw meat. Therefore, it was not possible for this work to establish a relevant association between these factors and recovery of *Campylobacter* spp. One cat from group H, being the only animal with access to the outside, had a complaint of chronic diarrhea, with all other animals being asymptomatic. Nonetheless, this animal's symptoms do not seem to fit a typical Campylobacteriosis description, because symptoms of this condition tend to be self-limiting, and none of the cohabitants display the same symptoms. The owner reports a frequent sharing of the food between this cat and a cohabitant dog, which could account not only for the symptoms, but also for isolation of *C. upsaliensis*, as opposed to more commonly recovered *C. helveticus*. While no *Campylobacter* spp. isolates could be obtained from the co-habitant dog, there is a possibility of contamination of the Blood Agar plate, and inability to separate pure *Campylobacter* spp. colonies from contaminants in this sample.

Considering the data obtained, it is not possible to establish a relevant association between recent antimicrobial treatment and isolation of *Campylobacter* spp. in this study. The same applies to consumption of raw meat. It would be interesting to perform future research on larger populations of sheltered animals, including, if possible, a greater amount of information on these animals' previous history, allowing more accurate comparisons between these animals and housed animals, as well as a more solid conclusions as to the influence of the living conditions in the frequency of *Campylobacter* spp isolation. It would also be relevant to collect more detailed information regarding history of gastrointestinal symptoms in the pets' owners, in order to associate these data with the isolates obtained

Regarding antimicrobial susceptibility testing, the resistances obtained were to ampicillin (33%), amoxicillin – clavulanate (50%), and tetracycline (33%). A study by Iannino and colleagues (2019), also performed disk diffusion test on *Campylobacter* spp. isolates and obtained a higher proportion of ampicillin resistance, with tetracycline resistance being 5% higher. However, the prevalence of amoxicillin – clavulanate resistant isolates obtained was higher in the present study. As for the remaining antimicrobial drugs tested, similarly low prevalences were obtained regarding erythromycin and gentamicin, while much higher prevalences were obtained by Iannino *et al.* for ciprofloxacin (Iannino *et al.*, 2019). Other studies have also reported high rates of resistance to tetracyclines and lower proportions of resistance to gentamicin and erythromycin (Carbonero *et al.*, 2012). A previous study also reports *C. jejuni* isolates to be more frequently resistant to β - lactam antibiotics, particularly ampicillin and amoxicillin, as well as tetracyclines (Aarestrup & Engberg, 2001). This information is, therefore, in line with the data obtained in the present work.

The lack of nitrocellulose membrane filters during part of the study caused heavily contaminated Blood Agar plates with possible loss of isolates, despite the subsequent cultures performed, in order to obtain pure colonies. Therefore, it is likely that this technical impairment resulted in a prevalence that is lower than the real prevalence among this population. It allowed, however, to conclude that isolation of pure *Campylobacter* spp. is much easier and accurate when a nitrocellulose filter is used, and this material is of critical importance when such studies are performed.

Despite the low prevalence obtained in this study, regarding particularly the presence of *C. jejuni* in dogs, and considering the resistances obtained to commonly used antimicrobials, the recovery and isolation of such bacteria represents, in itself, a potential threat to human health, which must not be disregarded. Future research, comprising larger populations of both animals and humans is recommended, in order to

continue the evaluation and monitoring of these bacteria in companion animals, and further associate its isolation with eventual risk factors and living conditions.

Conclusion

During my internship I performed four investigations, involving different animal populations (pets, bovines, and urban birds) and bacterial species. The common denominator was the interest in veterinary public health and the development of many different skills: collecting samples, performing epidemiological questionnaires, isolating and phenotyping *E. coli*, *Klebsiella* spp. and *Campylobacter* spp., determining antimicrobial resistance profiles and finally gaining experience in several molecular biology techniques.

Even so, it was possible to gather an interesting set of results that mostly concurs to the one obtained in previously published studies. Regarding colistin resistance, contrarily to what could be expected, and given the fact that antimicrobials are more frequently used in food-producing animals, the greater proportion of colistin resistant isolates was recovered from companion animals. While this may suggest a more accurate control of the antimicrobials that are administered to food animals, and the conditions in which they are administered, it is worrying that the larger number of colistin-resistant isolates was found in the population of animals that live closest to the humans. In addition, despite the fact that there was no previous history of colistin being administered to any of the animals included in the study, resistant isolates could still be recovered; while a co-selection process by other antimicrobial drugs is unlikely, it would still be of interest to further explore the genomic bases of colistin resistance and dissemination risk.

In what concerns the antimicrobial susceptibility of the isolates studied, a worrying number of MDR isolates was found in food producing animals, with the resistance profiles suggesting overuse of certain critical antimicrobial classes, such as fluoroquinolones. Unexpectedly, a large number of chloramphenicol-resistant isolates was recovered from fecal samples, which may suggest a cross-selection through other antimicrobial drugs. Under the One Health perspective, while these organisms, particularly the ones recovered from rejected milk, may not reach the consumer directly, their presence in farm animals is worrisome, due to the risk of indirect human exposure via food chain or environmental contamination.

Regarding companion animals, it is also relevant the fact that the greater number of resistant isolates, as well as the greater diversity of resistances, was found in sheltered animals, some of which may have ranged freely for undetermined periods of time. While these animals currently live in proximity with humans, and the presence of these isolates

is, in itself, a potential threat to human health, a number of these dogs and cats have also previously circulated through several environments, thus making it easier for these resistant microorganisms to spread to different locations. This suggests that it is important to continue investing on the monitoring, capturing, and rehousing of stray animals, and the data analyzed in this study may constitute a good starting point for further research on this particular group of animals. Although housed animals show a lesser number and diversity of resistance patterns – despite the fact that an ESBL-producing *E. coli* was isolated on this group - the presence of these isolates further highlights the importance of continued antibiotic stewardship and increased awareness on the matter, both from owners and veterinary professionals.

Finally, regarding *Campylobacter* spp., the presence of these zoonotic bacteria among animals so closely related to humans, propels further studies for monitoring this close human living animal population. While relevant associations between isolates and the living conditions of dogs and cats could not be established in this study, future research on the matter may be able to come to solid conclusions, regarding the living conditions and how these features can influence the recovery of resistant isolates.

Therefore, and always highlighting the importance of One Health, the findings of these preliminary studies further prove that the close proximity of animals and humans is not free of consequence; potential threats to human health, such as MDR bacteria and zoonotic pathogens are present in both food animals and companion animals, and must not be disregarded, but instead, continuously monitored and studied.

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Annex A

Questionário

Estudo *Enterobacteriaceae* + *Campylobacter*

(UpVet)

Identificação do Animal e Agregado

Nome: _____

Espécie: Canina/Felina

Sexo: F / M

Raça: _____

Concelho de residência: _____

Idade: _____

Ocupação dos tutores:

Coabitantes: S / N Quantos? Que espécies? 1 ☐ 2 ☐ > 2 ☐

Existem indivíduos vulneráveis no agregado familiar? (ex. idosos, grávidas, imunodeprimidos...):

Sim / Não

Existe história recente de sintomatologia gastrointestinal no agregado familiar? Sim / Não

Se sim, com diagnóstico? Sim / Não

O animal efetua deslocações? Sim / Não Para onde? ☐ 1 local ☐ 2 locais ☐ > 2 locais

Tempo decorrido desde a última estada: _____

Nesses locais, tem contacto com outros animais? Sim/Não

Ambiente

Tipo de ambiente: Interior ☐ Exterior Privado (Exclusivo) ☐ Exterior Público (Exclusivo) ☐ Misto

Se tem acesso ao exterior, que tipo de local frequenta? Rua ☐ Quintal ☐ Parque público ☐

Motivo: Passeio ☐ Necessidades fisiológicas ☐ Frequência: ☐ 1x/dia ☐ 2x/dia ☐ > 2x/dia

Tipo de abastecimento de água (consumo regular): ☐ Público ☐ Privado

Consumo ocasional de água em lagos, rios, charcos...? Sim ☐ Não ☐

Últimos 2 dias ☐ Última semana ☐ Último mês ☐ > 1 mês ☐

Alimentação

Se exclusivamente comercial: _____ % seca _____ % húmida

Se exclusivamente caseira, componentes maioritários:

Se mista: _____ % comercial _____ % caseira

Inclui (mesmo esporadicamente) carne crua? ☐ Sim ☐ Não

Passado Médico

Última toma de antibiótico (data): _____

Qual o antibiótico administrado? _____

Duração do tratamento: ☐ 7 a 15 dias ☐ 15 dias a 1 mês ☐ > 1 mês

Algun dos coabitantes foi medicado com antibiótico? ☐ Sim ☐ Não

Qual o antibiótico? _____

Há quanto tempo? Há < 1 semana ☐ Há 1 semana ☐ Há 1 semana a 1 mês ☐ Há > 1 ☐
mês

Annex B

Questionário – Estudo *Enterobacteriaceae* + *Campylobacter* (CRO)

Identificação do Animal:

Nome: _____ Espécie: Canina/Felina
Sexo: F / M Raça: _____ Idade: _____
Concelho: _____ Coabitantes: S / N (Espécie(s): Canina / Felina / Outro)

Ambiente

Uso frequente de antibióticos? Sim / Não
Algun dos coabitantes foi medicado com antibiótico recentemente? Sim / Não
Algun dos coabitantes tem alguma doença crónica? Sim / Não
Tipo de abastecimento de água: Público / Privado
O animal tem acesso a água do exterior (lagos, rios, charcos...)? Sim / Não

Tipo de alimentação

Se exclusivamente comercial: _____ % seca _____ % húmida

Se exclusivamente caseira, componentes maioritários:

Se mista: _____ % comercial _____ % caseira
Inclui (mesmo esporadicamente) carne crua? Sim / Não

Passado médico

Patologias relevantes:

Última toma de antibiótico (data): _____ / _____ / _____ Motivo:

Qual o antibiótico administrado? _____

Duração do tratamento: