

Relatório Final de Estágio
Mestrado Integrado em Medicina Veterinária

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Portugal – a One Health perspective**

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ABSTRACT

Campylobacteriosis has long been the most reported zoonotic disease in the European Union. As *Campylobacter* and their resistance genes can rapidly spread among human and animal populations, only a multisectoral *One Health* approach can tackle this problem. Since poultry is the main reservoir of *Campylobacter*, not much attention has been paid to other sources of infection, like companion animals. Despite these bacteria being mainly considered as commensal in dogs, the development of symptoms like those in humans, is also described. Given the public health and clinical practice concerns, and since no previous epidemiological study in Portugal was reported, we aimed to obtain a first insight of the prevalence and characteristics of this microorganism in different canine populations.

A total of 125 rectal swabs were collected from dogs held for companionship (n=71) and hunting (n=54), and from different regions: rural (n=75) and urban (n=50). Phenotypic characterization, including antimicrobial susceptibility testing (for *C. jejuni*, *C. coli* and *C. upsaliensis*), and genotyping through different molecular techniques was performed. Overall, 32 *Campylobacter* spp. isolates were obtained. *C. jejuni* (44%) and *C. lari* (41%) were the predominantly identified species, followed by *C. upsaliensis* (12%) and *C. coli* (3%). Antimicrobial resistance was found for all the three tested species. Regarding *C. jejuni* and *C. upsaliensis* isolates, the comparison of the phenotypic and genotypic traits with human isolates obtained in Portugal in the same year revealed a great similarity between both sources. This relationship was particularly relevant for *C. jejuni*, where the crossing of genome sequence data between both sources allowed the identification of isolates with high genetic proximity.

Despite being only a preliminary study, the close epidemiological relationship between the isolates obtained from both species revealed that dogs could be a more relevant source of *Campylobacter* to human than currently considered.

RESUMO

A campilobacteriose é, há muito, a doença zoonótica mais frequentemente reportada na União Europeia. A facilidade com que as bactérias e seus respectivos genes de resistência se podem transmitir entre as populações humana e animal exige uma abordagem integrada segundo a perspetiva “One Health”. Em virtude de as aves de capoeira serem consideradas o principal reservatório de *Campylobacter*, pouca atenção tem sido dada a outras fontes de infeção, tais como os animais de companhia. Nos cães, apesar destas bactérias serem principalmente consideradas comensais, o desenvolvimento de sintomas semelhantes aos do homem também está reportado. Atendendo às preocupações de saúde pública e prática clínica e dado não ter sido ainda publicado qualquer estudo epidemiológico em Portugal, neste trabalho pretendeu-se obter uma primeira perspetiva da prevalência e características deste microrganismo em diferentes populações caninas.

Um total de 125 zaragatoas retais foram colhidas de diferentes grupos de cães (companhia e caça), e de diferentes regiões (rural e urbana). Foi realizada a caracterização fenotípica, incluindo teste de suscetibilidade aos antimicrobianos (para *C. jejuni*, *C. coli* e *C. upsaliensis*), e a genotipagem por meio de diferentes técnicas de biologia molecular. Foi obtido um total de 32 isolados de *Campylobacter* spp. *C. jejuni* (44%) e *C. lari* (41%) foram as espécies predominantemente identificadas, seguidas por *C. upsaliensis* (12%) e *C. coli* (3%). Foi observada resistência aos antibióticos para as três espécies avaliadas. Relativamente aos isolados de *C. jejuni* e *C. upsaliensis*, uma comparação fenotípica e genotípica com isolados de humanos obtidos em Portugal no mesmo ano, revelou uma grande semelhança entre estes. Essa relação foi de particular relevância no caso de *C. jejuni*, onde o cruzamento de dados das sequências genómicas permitiu a identificação de isolados com alta proximidade genética.

Apesar de se tratarem apenas de resultados preliminares que carecem ainda de um estudo mais aprofundado, a estreita relação epidemiológica encontrada entre os isolados de ambas as espécies revelou que os cães podem ser uma fonte transmissora de *Campylobacter* para o homem mais relevante do que atualmente considerado.

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ABBREVIATIONS

AMC	Amoxicillin/clavulanic acid
AMP	Ampicillin
AMR	Antimicrobial resistance
AST	Antimicrobial Susceptibility Test
BA	Blood Agar
BIGSdb	Bacterial Isolate Genome Sequence Database
CC	Clonal Complex
CN	Gentamicin
CP	Ciprofloxacin
COS	Columbia agar
DNA	Deoxyribonucleic acid
ECDC	<i>European Centre for Disease Prevention and Control</i>
EFSA	<i>European Food Safety Authority</i>
ERIC-PCR	Enterobacterial repetitive intergenic consensus Polymerase Chain Reaction
E	Erythromycin
eMLST	Extended Multilocus Sequence Typing
ETP	Ertapenem
EU	European Union
FAO	Food and Agriculture Organization
MALDI-TOF	Matrix assisted laser desorption ionization–time of flight
MLST	Multilocus Sequence Typing
MST	Minimum spanning tree
OHEJP	One Health European Joint Programme
OIE	World Organisation for Animal Health
PCR	Polymerase Chain Reaction
PT	Portuguese
ST	Sequence Type
TE	Tetracycline
UPVET	Veterinary Hospital of the University of Porto
UPW	Ultrapure Water
wgMLST	Whole Genome Multilocus Sequence Typing
WGS	Whole Genome Sequencing
WHO	World Health Organization

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1 - INTRODUCTION

As the oldest domesticated animal, a dog is wherever a man is. Though companionship is the main purpose of a dog in the western world, there are still many significant roles they play in our society, as dogs can assist humans in several activities such as hunting, guarding, herding or be used as service and assistance dogs (Hart & Yamamoto, 2016; Macdonald & Carr, 2016; Mariti et al., 2013; Vonholdt & Driscoll, 2016). Humans can greatly benefit from this relationship; adding to the previously mentioned reasons, the prophylactic and therapeutic value that human–dog bond can have in people's health has been increasingly studied, and their positive impact has undoubtedly been demonstrated (Wells, 2007; WHO, 2013). Like in every relationship, there are also great costs to this equation. Not only do dogs threaten global health through attacks to either people, other pets, livestock or wildlife, but also the zoonotic diseases they transmit and its high impact in public health is a major reason of concern (Macpherson, 2013; Wells, 2007; WHO, 2013). In fact, dogs have been associated with the transmission and perpetuation of over 65 zoonotic agents including bacteria, protozoa, ectoparasites and helminths (Macpherson, 2013). This is a subject of special matter in rural regions, where the close relationship between domestic dogs, livestock, wildlife, and the ecosystems, constitutes a major bridge between them all, reminding us of the need to address global health as one.

The awareness that animal and human health are closely related and have a common interaction with the environment has been present in the scientific community for centuries and led, over time, to the introduction of the concept of "One Health" in 2008 (FAO et al., 2008; Kahn, 2017). This concept aims to eliminate the artificial barriers created between the different areas of public health, thus obtaining a more robust and effective collaborative force in overcoming the health risks present at the human and animal ecosystems interface (FAO et al., 2008; Monath et al., 2010).

With 60% of human infectious diseases originating from domestic animals and wildlife, and with 75% of human infectious diseases emerged or re-emerged in the last decades being zoonoses, veterinary science has a vital role in the "One Health" approach (FAO et al., 2008; OIE, 2021; Woolhouse & Gowtage-Sequeria, 2005). Early detection and control of infectious diseases in their animal source can prevent their transmission to humans and, as such, is the most economical and efficient way to protect human health (OIE, 2018, 2021). In order for it to be a union of forces and not just different parts fighting for a common cause, strong communication and collaboration efforts between the different sectors involved is mandatory. Worldwide, FAO, OIE and WHO have formed a tripartite alliance to coordinate and promote intersectoral collaboration in obtaining the necessary

data and knowledge on infectious diseases, as well as to share the responsibility for promoting Global Health (FAO et al., 2008; WHO, 2020). In the EU, to strengthen the efforts already employed by EFSA and ECDC, the One Health European Joint Programme (OHEJP) was established in 2018. This program has the collaboration of 44 food, veterinary and medical laboratories and institutes, many of them with reference responsibilities across 22 member states. This solid unit aims to harmonize methods, databases and procedures for the evaluation and management of the risks associated with zoonoses. With a clear purpose of sharing knowledge and experiences that facilitate the achievement of collaborative solutions, OHEJP has several joint research projects, PhD projects, workshops and scientific meetings regarding the containment of emerging One Health problems (Brown et al., 2020; One Health EJP, 2019).

Within public health, special attention must be given to Antimicrobial Resistance (AMR), as it constitutes a serious threat to the achievements modern medicine has made to successfully treat bacterial infections (WHO, 2020). Like any other selective pressure, antibiotics promote an evolutionary pressure in bacteria directed towards greater adaptation and survival to the surrounding environment; thus, the abuse or misuse of antimicrobial drugs highly contributes to the resistance phenomenon. As extensively known, bacteria take advantage of their genomic flexibility for the acquisition and expression of resistance genes, either through *de novo* mutations in chromosomal genes or through acquisition of horizontally transferred resistance determinants (Kahn, 2017; Palma et al., 2020). Thus, the ease with which bacteria and their resistance genes can spread among human and animal populations means that the pressure applied in one sector is reflected in the others, which forces a broader approach when facing this problem (McEwen & Collignon, 2018).

In fact, the field most frequently associated with the misuse of antibiotics and consequent promotion of microbial resistance is veterinary practice, mainly animal production, where only after many years of disproportionate use of antibiotics, particularly as growth promoters, the risks that these practices poses to man have been raised (Shah et al., 1993).

Campylobacteriosis is, as has been since 2005, the most reported zoonotic disease in the EU (EFSA, 2019). With symptoms that typically appear after an incubation period of two to five days, it usually consists of a watery or bloody self-limiting diarrhea, abdominal pain and fever, and more severe manifestations are rarely observed (Barrett & Fhogartaigh, 2017; Rao et al., 2001). Although it typically does not require special treatment and the use of antibiotics is not recommended, in some cases, the persistence of symptoms requires antibiotic therapy and hospitalization, especially in immunocompromised patients. Not often, gastroenteritis is followed by chronic complications, such as reactive arthritis,

Guillain-Barré and Miller-Fisher syndromes (Islam et al., 2009; McGrogan et al., 2009; Revez et al., 2011; Takahashi et al., 2005).

Campylobacter spp. is a Gram-negative slender, curved or spiral rod, with dimensions ranging from 0.5 to 8.0 µm long and 0.2 to 0.5 µm wide (Goni et al., 2017; Willey et al., 2008). It presents a spiral movement produced by the uni or bipolar flagella present in one or both ends of the bacterium, respectively. Usually positive to oxidase and catalase test, it grows at temperatures between 37° and 42° C under microaerophilic conditions (Willey et al., 2008).

Among the 32 species described to date, *Campylobacter jejuni*, *Campylobacter coli*, *Campylobacter lari* and *Campylobacter upsaliensis* are the pathogens most frequently associated with campylobacteriosis in humans (Costa & Iraola, 2019; EFSA, 2019; Iannino et al., 2019). Although in most cases the causative agent is *C. jejuni*, an underestimation of the role that other emerging species of *Campylobacter* play has been recognized, and the need for further study on pathogenicity, transmission and evolution of these non-*jejuni*, non-*coli* *Campylobacter* species is undeniable (Costa & Iraola, 2019; Iannino et al., 2019; Man, 2011; WHO, 2012).

Different techniques can be used when identifying *Campylobacter* spp.; since traditional biochemical assays are quite fallible and limited (allowing at most genus identification), further analysis with higher accuracy methods is usually needed.

Multiplex PCR is a technique first described by Chamberlain and coworkers (1988) that allows simultaneous amplification of several DNA sequences in a single PCR reaction. When applied to the identification of *Campylobacter*, this technique uses different primers aiming at the main species, allowing the simultaneous genus confirmation and species identification (provided that the species is within the aim of the multiplex). Given the usefulness of this technique regarding *Campylobacter*, several multiplex protocols have already been developed and are regularly used (Klena et al., 2004; Wang et al., 2002; Yamazaki-Matsune et al., 2007).

Matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry is a technique that uses ionization and desorption of molecules for biomolecular analysis (Jurinke et al., 2004). When applied to microbiology, it allows the analysis of the protein composition of bacterial cells (Public Health England, 2018). The measurement of the time needed for ions to reach the receptor, allows the measurement of their mass and subsequent species identification (Jurinke et al., 2004). It is a technique with high sensitivity and reproducibility, that allows a rapid identification of *C. jejuni* and *C. coli*, but also numerous emerging *Campylobacter* species due to consistent efforts towards optimization (Bessède et al., 2011; Mandrell et al., 2005; Public Health England, 2018).

The identification of the species involved in the infection is only the beginning of the agent characterization, as within the same bacterial species, there are organisms with a great diversity of characteristics. The identification of this variety is called typing and is essential for public health surveillance and outbreak response (MacCannell, 2013; Sabat et al., 2013). Phenotyping is the characterization of an organism based on their expressed traits. Antimicrobial susceptibility testing, serotyping or bacteriophage typing, are examples of phenotyping techniques that have been used for many years. Despite the ease of use and interpretation, of being generally accessible and practical techniques, the low discriminatory power and low reproducibility, limits the value of this kind of technique in epidemiological investigations (Farber, 1996; Ranjbar et al., 2014).

Over time, the development of molecular biology revolutionized the ability to distinguish different types and subtypes of bacteria, as it allowed DNA-based typing: genotyping (Sabat et al., 2013). With an increasing number of techniques being developed, choosing the most appropriate genotyping method is not always simple, and characteristics such as reproducibility, discriminatory power, affordability and ease to perform should be taken into account (MacCannell, 2013; Ranjbar et al., 2014).

Enterobacterial repetitive intergenic consensus-PCR (ERIC-PCR) is a profile-based technique that involves gel-based fragment analysis. Based on the presence of highly conserved repeats of DNA sequence elements throughout the genome of many enteric bacteria, this technique uses consensus primers designed to expand the DNA sequences present between two successive repetitive elements (Versalovic et al., 1991). The variability of the position of these elements along the genome of different species and strains, allows amplicons of different dimensions to be obtained, which, after separation by electrophoresis, form a characteristic band profile that can be compared to that of other isolates (Foley et al., 2009; Ranjbar et al., 2014; Sabat et al., 2013). This is a simple, very reproducible method, with a moderate discriminatory power (Farber, 1996). Its usefulness regarding foodborne pathogens is well described, particularly in multiresistant *Enterobacteriaceae* strains (Foley et al., 2009; Ranjbar et al., 2014). Regarding *Campylobacter*, although there is still not a clear validation of the technique, some studies point out the usefulness of ERIC as a typing method (Ahmed et al., 2015; Aquino et al., 2010; Moser et al., 2001).

On another hand, multilocus sequence typing (MLST) is a genotyping technique that, rather than relying in DNA fragment size, it relies in nucleotide base changes associated to genetic drift (Ranjbar et al., 2014) among six to eight housekeeping genes. (Foley et al., 2009; MacCannell, 2013; Ranjbar et al., 2014). MLST has an online database (<https://pubmlst.org/>) where, for each locus, users can upload their sequence that will be compared with those already uploaded to the system. If the allele corresponding to that

sequence has previously been introduced in the database, the same number will be associated with the isolate, if not, a new number will be assigned. In the end, to the combination of identified alleles, a sequence type (ST) is associated. Isolates from different STs that have great similarity to each other (*i.e.* six of seven identical alleles) are assigned to the same clonal complex (CC) (Foley et al., 2009; Sabat et al., 2013). Regarding *Campylobacter*, this technique uses *aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *tkt*, and *uncA* loci for typing isolates, and its usefulness is well described (Foley et al., 2009; Lévesque et al., 2008; Rowe & Madden, 2014). To increase the discriminatory power, which often becomes limited when only seven loci are analyzed, different variations of this technique, comparing a larger number of loci have emerged, such as extended MLST (eMLST) (based on the comparison of 21 loci), reaching over 2000 compared loci in the case of whole genome-MLST (wgMLST). In this case, data on whole genome must be available, which is achieved by whole genome sequencing (WGS), a revolutionizing technology that allows a comprehensive analysis of bacterial genome and whose discriminatory power, capable of distinguish even highly related strains, finds its most extensive usefulness in the epidemiological studies of foodborne pathogens (Besser et al., 2018; Uelze et al., 2020). The fact that WGS provides the entire genome allows, without any added effort, the comparison of an infinite number of nucleotide sequences, as is the case of (*flaA* svr), a short variable region (SVR) within the flagellin A coding sequence, whose usefulness in *Campylobacter* typing has long been described (Meinersmann et al., 1997).

Despite the total objectivity of the results, which with the database and online resources available are easily compared worldwide, WGS high price and the need of technically advanced equipment for interpretation, makes it unsuitable for routine use in surveillance or outbreak investigation (MacCannell, 2013; Ranjbar et al., 2014; Rowe & Madden, 2014).

In summary, no typing method alone is perfect or even sufficient; therefore, a wise choice of pheno- and genotyping techniques applied in epidemiological research is vital for an efficient characterization of outbreak investigation and source of infection determination.

As previously stated, human campylobacteriosis is mainly a foodborne disease, frequently associated with the consumption and handling of contaminated meat. Although also associated with livestock, poultry is its main reservoir, being responsible for 80% of human infections (EFSA, 2011; Inglis et al., 2004; Mullner et al., 2009; Newell et al., 2001). In addition, other sources of infection such as wild animals, environmental water and pets have been described and should also be considered (EFSA & ECDC, 2020; WHO, 2012). With approximately 6% of *Campylobacter* infections in humans being caused by contact with companion animals, owning a dog, particularly puppies, has been also described as a risk factor for *C. jejuni* and *C.coli* infection, with even outbreaks linked to puppy exposure

being described (Gras et al., 2013; Iannino et al., 2019; Montgomery et al., 2018). *C. upsaliensis* has dogs and cats as its main reservoir, and despite being the prime suspect in undiagnosed cases of human gastroenteritis, its prevalence and clinical relevance is considered to be highly underestimated (Bourke et al., 1998; Man, 2011; Vandenberg et al., 2006).

In dogs, *Campylobacter* is considered mainly as a commensal bacteria and the predominantly found species are *C. jejuni* and *C. upsaliensis*, with a prevalence of around 50% among asymptomatic dogs (Acke, 2018; Acke et al., 2009; Iannino et al., 2019). Despite this fact, this bacterium is also described as pathogenic in dogs and is associated with symptoms similar to those described in humans, with watery or bloody mucoid diarrhea, anorexia and fever, especially in puppies (Acke, 2018; Marks et al., 2011). Extra-intestinal manifestations like abortion have also been associated with *Campylobacteriosis* in dogs, something commonly found in ruminants (Bulgin et al., 1984; Odendaal et al., 1994; Sahin et al., 2014). Recent studies also described a possible association between *C. jejuni*, *C. upsaliensis* and acute polyradiculoneuritis, an immune-mediated neuropathy in dogs, which is similar to Guillain-Barré syndrome in humans (Martinez-Anton et al., 2018).

Although the veterinary focus of "One Health" has been mainly livestock and wildlife, pets, and particularly dogs, with their ever closer contact with humans and being able to transmit countless zoonoses, including resistant bacteria, play an important role in the challenges that infectious diseases represent to public health (Guardabassi et al., 2004; Overgaauw et al., 2020). Thus, much more studies are needed in order to elucidate the importance of dogs in the transmission of infectious bacteria relevant to public health.

2 - AIM OF THE STUDY

Regardless of the uncertainty about the clinical relevance of *Campylobacter* in dogs, these companion animals can be reservoir of a wide variety of species, that can be transmitted to humans and other animals (Chaban et al., 2010; EFSA, 2008). Therefore, since there was no previous epidemiological study regarding *Campylobacter* spp. in dogs in Portugal, the present study aimed to obtain a first insight of the prevalence and characteristics of this microorganism in different canine populations. Taking into account the concerns of veterinary clinical practice and public health, its phenotypic characterization, including antimicrobial susceptibility testing and genotypic characterization through comparison of different molecular techniques was intended.

3 - MATERIALS AND METHODS

3.1 - SAMPLE SELECTION

Two main groups of samples were initially established. The first group, group A, consisted of 50 rectal swab specimens obtained from dogs presented at the Veterinary Hospital of the University of Porto (UPVET), between September 22 and December 2, 2020. A random sample of patients was selected among the animals admitted to hospital (n=15), pre- and post-surgery patients (n=14) and animals presented for consultation (n=21). This study was approved by the *Organismo Responsável pelo Bem-estar dos Animais do Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto* (ORBEA ICBAS-UP) and along with a written consent for participation in the study, information about animal age, sex, breed, cause of the visit to the hospital, signs of gastrointestinal illnesses, general living environment, and any recent antibiotic treatment was obtained from the animals' owners through a short questionnaire. Of the 50 dogs (male n=21; female n=29), 9 had a recent history of diarrhea, 4 of them were in the hospital for that reason.

The second group, group B, was composed by the samples obtained from boarhounds (wild boar hunting dogs) from Monção, a rural area in the north of Portugal. Two weeks after the opening of the boar hunting season, 17 (male n=9; female n=8) rectal swabs were collected from a boarhound pack. Concerning this group, 12 had already been hunting that season and five had not still. A month after the first sampling campaign, after all the animals had been hunting in "montarias" (typical Iberian boar driven hunt), a second swab was collected from 15 of the initial 17 dogs sampled. Along with this boarhound pack, 22 (male n=14; female n=8) samples from small game hounds from the same area were obtained from three different packs (n=8; n=11; n=3), totaling 54 specimens from group B. Regarding this group, none of the animals presented history of gastrointestinal disease.

Due to the disparity of the number of *Campylobacter* isolates obtained in the two groups, and in an attempt to understand whether this difference was related to geographic region or the purpose of the animal (hunting dog vs companion dog), a third, smaller group (group C) was created, consisting of 21 samples (male n=11; female n=10) collected in a veterinary clinic placed in the same rural area as group B. In three consecutive Fridays (30/10, 06/11 and 13/11, 2020), samples were collected with the exact same criteria of selection as used in group A, along with the same inquiry form. From this cluster, two were post-surgery patients, one was an inpatient and the remaining (n=18) were animals presented for consultation. Only three dogs presented a recent history of diarrhea.

3.2 - SAMPLE IDENTIFICATION

An identification code (ID) was assigned to each animal, combining the letter of their group with a number correspondent to the sampling order within the group (*i.e.*, to the first dog to be analyzed in group C was assigned the ID C1). Each sample was identified with the same ID as the dog to which it belonged. In the second sampling campaign of group B, the same number was given to the same animal and the sample was identified as Br(n) (*i.e.*, Br9: second sample from the B9 dog).

When more than one strain was obtained from the same sample, a second number was added to the previous one (*i.e.*, Br2/1 and Br2/2: two isolates from sample Br2).

3.3 - CULTURE AND ISOLATION OF *CAMPYLOBACTER* SPP

Immediately after the smear was performed, swabs were placed into 10 mL of *Bolton Broth* (*Bolton Broth*, Oxoid with 5% Horse blood Lysed, Oxoid). Inoculated broths were incubated for 48 h, at 41.5 °C under microaerophilic conditions generated by CampyGen™ (Oxoid). After the enrichment, 120 µL of the suspension were placed in BA (Blood Agar: Tryptone-casein soy agar, Biokar diagnostics + 5% Horse Lysed blood, Oxoid) through a 0.65 µm membrane filter (*Nitrocellulose membrane filters*, Whatman) and subsequently incubated in the same conditions, for another 48h period (ISO 10272:2006-1).

Campylobacter-like colonies detected after incubation were oxidase tested (Oxidase Test Stick, Liofilchem), and their morphology and motility evaluated through phase-contrast microscopy. When the observed features were indicative of *Campylobacter* spp. (oxidase positive, spiral-shaped bacteria and characteristic motility), a subculture in BA was performed in duplicate, under both micro and aerophilic conditions for 48h, 41.5°C. If growth was observed only in the plate incubated in microaerophilic conditions, DNA was extracted through a suspension of culture material in 20 µL TE buffer (Tris 10mM + EDTA 1mM, pH=8). After an incubation of 15 min at 95°C, 180 µL of Ultrapure Water (UPW) was added and the mixture centrifuged at 12000 rpm for 3 min. The sediment was discarded, and the supernatant preserved.

3.4 - ISOLATE IDENTIFICATION

A PCR Multiplex *Campylobacter* was performed as described by Yamazaki-Matsune and colleagues (2007) for genus confirmation and species identification. Along with *Campylobacter* spp, this multiplex PCR assay was targeted to *C. jejuni*, *C. upsaliensis*, *C. coli*, *C. fetus*, *C. lari* and *C. hyointestinalis*. Using the same primers and reaction conditions as the ones described by the author, the PCR products were analyzed by gel

electrophoresis through a 1.5% (weight/volume) agarose (Agarose Ultrapure grade, NZYtech) in 1X TBE buffer, stained with 1% GreenSafe (GreenSafe Premium, NZYTech).

All the isolates confirmed as *Campylobacter* spp. by the Multiplex PCR, but whose species could not be identified by this method, were submitted to identification by matrix assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (Vitek® MS).

3.5 – ANTIMICROBIAL SUSCEPTIBILITY TESTING

Antimicrobial susceptibility testing was performed, according to the Kirby-Bauer method, in every isolate identified as either *C. jejuni*, *C. coli* or *C. upsaliensis*.

In Mueller-Hinton agar supplemented with 5% defibrinated horse blood (Oxoid) and β -NAD (Sigma), a 0.5 McFarland suspension of bacterial culture in saline solution was inoculated and antimicrobial susceptibility testing was performed by disk diffusion (charge of the disk), for the following drugs: ciprofloxacin (CIP) (5 μ g), erythromycin (E) (15 μ g), tetracycline (TE) (30 μ g), gentamicin (CN) (10 μ g), ampicillin (AMP) (10 μ g) amoxicillin/clavulanic acid (AMC) (30 μ g) and ertapenem (ETP) (10 μ g) (Oxoid).

The diameters of the inhibition zones were measured after a 24h incubation, at 41.5 °C, in a microaerobic environment generated by Anoxomat (Advanced Instruments Inc.) Results were interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) and *Comité de l'antibiogramme de la Société Française de Microbiologie cut-offs*.

3.6 – MOLECULAR TYPING

Molecular typing of *C. jejuni*, *C. upsaliensis* and *C. lari* was performed using enterobacterial repetitive intergenic consensus PCR (ERIC-PCR). After the bacterial pellet was suspended in 200 μ L of eMag Lysis buffer, DNA was extracted in the automated nucleic acid extraction platform eMAG™ (bioMérieux). Using the ERIC motifs designed by Versalovic and colleagues (1991), ERIC-1R (5'-ATGTAAGCTCCTGGGGATTAC-3') and ERIC-2 (5'- AAGTAAGTGACTGGGGTGAGCG-3'), each 25 μ L reaction mixture contained 10 μ L of HotStarTaq Plus Master Mix (Qiagen), 4.2 mM MgCl₂, 0.2 μ M of each primer and 3 μ L of template DNA. After an initial incubation at 95°C for 5 min, 40 amplification cycles (94 °C for 1 min, 25°C for 1 min and 72°C for 2 min) were performed, followed by a final elongation at 72 °C for 10 min. By electrophoresis, the PCR products were separated in 2% (w/v) agarose (SeaKem LE Agarose, Lonza) in 0.5X TBE buffer, stained with GelRed (Sigma Aldrich) for approximately 2 h.

3.7 – WHOLE GENOME SEQUENCING (WGS)

Whole genome sequencing (WGS) was performed in some selected isolates of *C. jejuni*, using the Illumina technology. *In silico* Multilocus Sequence Typing (MLST), extended Multilocus Sequence Typing (eMLST) and *flaA* short variable region (*flaA* svr) typing was determined at PubMLST platform (<https://pubmlst.org/>).

After *de novo* assembly with INNUca v3.1 pipeline (<https://github.com/B-UMMI/INNUca>), analysis was performed using the wgMLST scheme for *C. jejuni* provided by INNUENDO and the phylogenetic relationship (based on allelic differences) between the isolates was represented using the online platform PHYLOVIZ online 2.0 Beta version (<http://online2.phyloviz.net/>).

4 – RESULTS

From a total of 125 rectal swabs, 26 (21%) had positive culture for *Campylobacter* spp. and 32 isolates were obtained. It can be seen from the data in figure 1 that most isolates came from group B (25/32), the group of hunting dogs. Despite having less than a half of the samples, the number of isolates obtained from group C (veterinary clinic) was very similar to the one found in group A (veterinary hospital), with 3 and 4 isolates respectively. Regarding the distribution of *Campylobacter* species among colonized animals, *C. jejuni* (n=14) and *C. lari* (n=13) were the predominant species, followed by *C. upsaliensis* (n=4) and *C. coli* (n=1). Mixed colonization by *C. jejuni* and *C. lari* was detected in five dogs; in another dog, two different *C. jejuni* isolates were obtained.

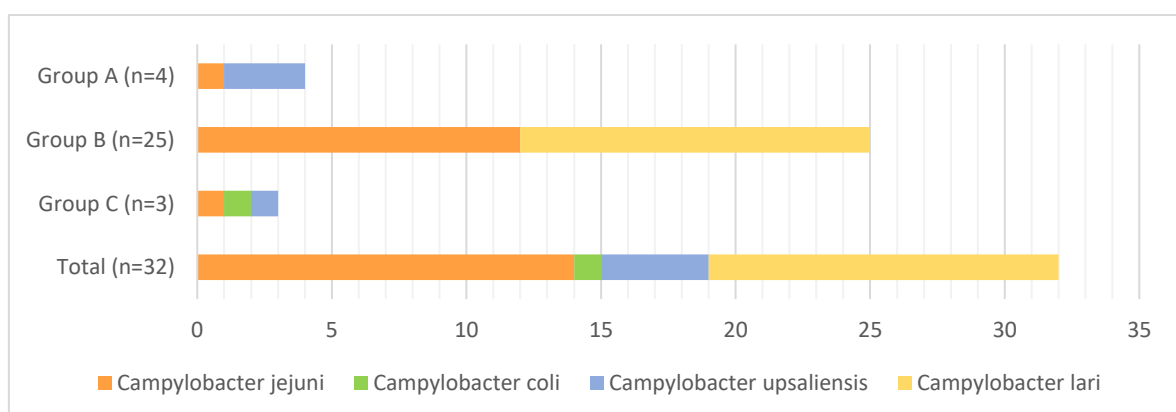


Figure 1: Distribution of *Campylobacter* species among the isolates of each group (n=32). Group A: Veterinary Hospital (number of samples=50); Group B: hunting dogs (number of samples=54); Group C: Veterinary Clinic (number of samples=21).

Information stratified by age, sex, and presence of clinical signs, regarding groups A and C is presented in Tables 1 and 2, respectively. From the animals examined at the

veterinary hospital (group A), three isolates of *C. upsaliensis* and one of *C. jejuni* were obtained, all of them from diarrheal animals. On the contrary, regarding group C (veterinary clinic), none of the animals carrying *Campylobacter* spp. had diarrhea, with *C. jejuni*, *C. upsaliensis* and *C. coli*, one strain of each, being detected in three different dogs.

Table 1: Distribution of *Campylobacter* species in group A-veterinary hospital (n=50 samples)

Variable	Category	n. of samples	<i>C. jejuni</i>	<i>C. upsaliensis</i>	<i>Campylobacter</i> spp.
Age	0-2	16	1	2	3
	>2-4	6	0	0	0
	>4-8	9	0	0	0
	>8	19	0	1	1
Sex	Male	21	1	1	2
	Female	29	0	2	2
Diarrhea	Yes	9	1	3	4
	No	41	0	0	0
Motive	Surgery	14	0	1	1
	Inpatient	15	0	2	2
	Consultation	21	1	0	1

Note: In many cases, data about recent antibiotic treatment was not available. In addition, considering the irrelevant complexity regarding the breeds of the animals, these variables were not presented.

Table 2: Distribution of *Campylobacter* species in group C-veterinary clinic (n=21 samples)

Variable	Category	n. of samples	<i>C. jejuni</i>	<i>C. coli</i>	<i>C. upsaliensis</i>	<i>Campylobacter</i> spp.
Age	0-2	9	0	1	0	1
	>2-4	2	1	0	0	1
	>4-8	4	0	0	1	1
	>8	6	0	0	0	0
Sex	Male	11	1	1	1	3
	Female	10	0	0	0	0
Diarrhea	Yes	3	0	0	0	0
	No	18	1	1	1	3
Motive	Surgery	2	0	0	0	0
	Inpatient	1	0	0	0	0
	Consultation	18	1	1	1	3

Note: In many cases, data about recent antibiotic treatment was not available. In addition, considering the irrelevant complexity regarding the breeds of the animals, these variables were not presented.

Regarding group B, most of the *Campylobacter* isolates were obtained from the boarhound pack (n=23) with only two samples of *C. jejuni* being isolated from small game hounds (both belonging to the same pack). The distribution of the small game samples is shown in Table 3. As a different approach was used with boarhounds, with a second sampling event taking place after all the dogs have gone hunting (a month later), information that compares the results of both moments is presented in Table 4. A second smear from

dogs B1 and B16 was not performed since they were not present in the kennel at the time of collection. There was a trend for the same dogs to have the microbiological culture positive for *Campylobacter* spp. in both sampling points. Only from two animals with a positive sample in the first sampling point, no isolate was obtained in the second; also, only a previously negative dog, tested positive in the second smear. It should be noted that, in the second collection, most of the animals presenting *Campylobacter* had a mixed colonization (6/8), a fact not observed in the first one.

Table 3: Distribution of *Campylobacter* species in small game hounds from group B (n=22 samples)

Variable	Category	N. of samples	<i>C. jejuni</i>
Age	0-2	10	1
	>2-4	9	1
	>4-8	3	0
	>8	0	0
Sex	Male	14	1
	Female	8	1
Recently hunting	Yes	19	1
	No	3	1
Pack	1	8	2
	2	11	0
	3	3	0

Table 4: Summary of the results from both sampling events from the boarhounds (n=17 dogs)

Dog	Sex	Age	First sampling point		Second sampling point	
			Recently hunting	<i>Campylobacter</i> species	Recently hunting	<i>Campylobacter</i> species
B1	F	0-2	No	Ø	^a	^a
B2	M	>4-8	Yes	<i>C. lari</i>	Yes	<i>C. jejuni</i> + <i>C. lari</i>
B3	M	>8	Yes	<i>C. jejuni</i>	Yes	<i>C. lari</i>
B4	F	>4-8	No	Ø	Yes	<i>C. jejuni</i> + <i>C. lari</i>
B5	F	>2-4	Yes	<i>C. lari</i>	Yes	<i>C. jejuni</i> + <i>C. lari</i>
B6	F	0-2	No	<i>C. lari</i>	Yes	<i>C. jejuni</i> + <i>C. lari</i>
B7	M	0-2	Yes	<i>C. lari</i>	Yes	<i>C. jejuni</i> + <i>C. lari</i>
B8	M	>2-4	Yes	<i>C. lari</i>	Yes	<i>C. jejuni</i> + <i>C. jejuni</i>
B9	M	>2-4	Yes	<i>C. jejuni</i>	Yes	<i>C. jejuni</i>
B10	M	0-2	Yes	Ø	Yes	Ø
B11	F	>2-4	Yes	Ø	Yes	Ø
B12	F	>2-4	Yes	Ø	Yes	Ø
B13	F	0-2	No	<i>C. lari</i>	Yes	Ø
B14	M	>2-4	Yes	Ø	Yes	Ø
B15	M	0-2	Yes	Ø	Yes	Ø
B16	M	0-2	Yes	Ø	^a	^a
B17	F	>2-4	Yes	<i>C. lari</i>	Yes	Ø

Ø No *Campylobacter* spp. isolate was obtained in this sample.

^a Dog not present in the kennel at the time of collection, smear not performed.

Although a substantially higher number of isolates was obtained from group B, considering the numerous bias present in this study (animals from the same household, second sampling from the same animal, etc.), prevalence studies were not carried out, and the study was conducted with a qualitative and prospective purpose. Therefore, in order to facilitate the exposition of the results and allow a more direct comparison, results were presented by *Campylobacter* species.

4.1 – *CAMPYLOBACTER JEJUNI*

Of the total *C. jejuni* isolates (n=14), the PCR Multiplex was only able to identify seven to species-level, simply recognizing the remaining seven as *Campylobacter* spp.. Further identification was then carried out by MALDI-TOF. Antimicrobial susceptibility testing was performed to every *C. jejuni* strain and the results are summarized in Figure 2. A considerable proportion of the *C. jejuni* isolates displayed resistance to CIP (93%), TE (64%) and AMP (57%). No resistance to E, CN, AMC acid or ETP was found. Altogether, 5 different drug resistance profiles of *C. jejuni* were found. Three isolates were multidrug resistant, presenting resistance to CIP, TE and AMP; five were simultaneously resistant to CIP and TE, and four to CIP and AMP; one isolate showed to be resistant to TE and AMP and another one only to CIP.

Some technical issues delayed the recovery of isolate C3 (veterinary clinic of Monção), and it was not possible to include it in further genotypic analysis.

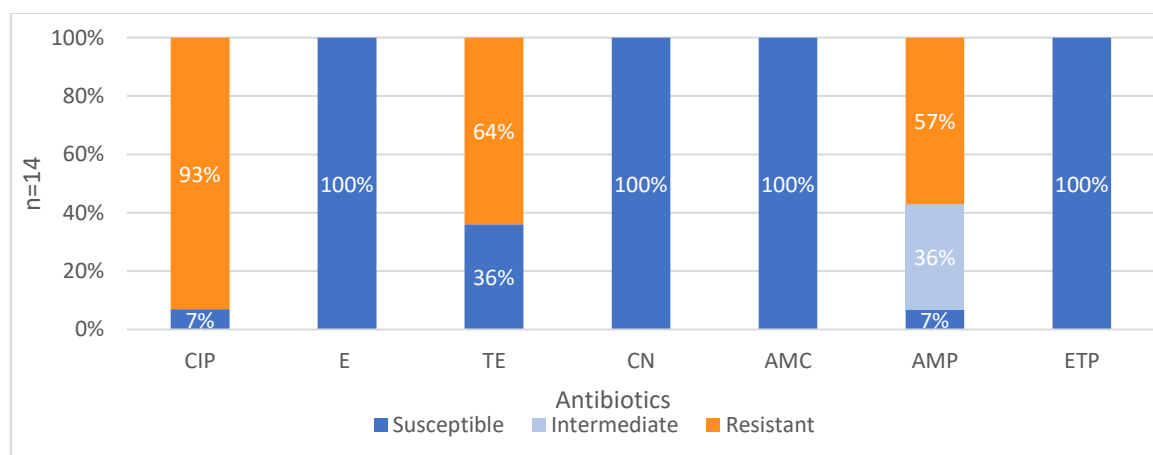


Figure 2: Antimicrobial susceptibility (%) of *Campylobacter jejuni* isolates from dogs (n=14).

ERIC-PCR was tested in this species with the first results showing clearly different profiles, thus suggesting the presence of different strains (Figure 3). In fact, among 13 isolates tested, seven different profiles were obtained. When, among different profiles, the similarities between them were obvious and only in a small number of bands differences were observed, the isolates were considered as belonging to the same cluster. Thus, the

isolates were grouped into 4 clusters. The profile of B3, obtained in the first sampling event of the boarhounds, showed no similarity to any other profile and as such it was assigned to an independent cluster (cluster 1, C1 in Figure 3). Three samples of group B were assigned to cluster 2 (C2): B9, an isolate found in the first sampling event in boarhounds and whose profile was slightly different to the other two, B19 and B23, which belonged to the *C. jejuni* isolated from the same pack of small game hounds. Cluster 3 (C3) consisted of three isolates obtained in the second sample collection of boarhounds, two of which (Br2/1 and Br8/1) with the same profile and the other, Br7/2 with a slightly different fingerprint. Finally, cluster 4 (C4), the largest one, consisted of six samples: five of them were isolates from the second swabs performed on the boarhounds (Br4/2; Br5/1; Br6/1; Br8/2 and Br9) and the sixth one belonged to the *C. jejuni* isolated at the veterinary hospital in Oporto (A11). This last cluster was the one presenting the greatest homogeneity in terms of profiles, with only one isolate (Br8/2) presenting an extra band in relation to the others. Curiously, none of isolates from cluster 4 were identified by PCR-multiplex at species level.

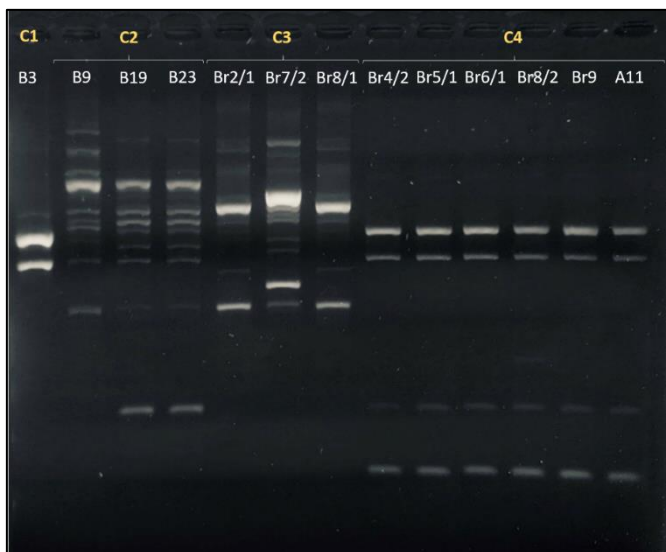


Figure 3: ERIC-PCR fingerprints of *Campylobacter jejuni* isolated in dogs. The ID of the sample is presented in white. The letter and numbers in yellow represent the cluster assigned to profile.

In order to further explore genetic diversity, 10 isolates representative of the variety of profiles obtained were sent to WGS. These strains were also subjected to MLST typing and *flaA* svr. In general, results obtained were highly consistent with those obtained by ERIC-PCR (Table 5); in fact, each cluster corresponded to a specific ST, with the exception of C2, which hosts two different STs: strain B9 belongs to ST-8569 and B19 to ST-148. All sequenced isolates from cluster 3 belonged to ST-6461, whereas cluster 4 isolates were assigned to ST-22. Isolate B3, the only member of cluster 1, belonged to ST-48. ST22 and ST 6461 were the most frequently found genotypes in the group of dogs studied. Regarding

flaA svr, the results appeared to be in accordance with the previous typing methods, as the isolates belonging to the ST-22 (the group that seemed to have the greatest phylogenetic proximity), all had the same *flaA* svr genotype (*flaA*-161), and among those belonging to the ST-6461 (cluster that displays slight differences regarding ERIC-PCR profiles), a *flaA* svr variety was observed, with the isolate Br2/1 belonging to *flaA*-67; Br7/2 to *flaA*-49/395 and Br8/1 to *flaA*- 49.

Table 5: ERIC-PCR, ST, CC and *FlaA* svr of the group of *Campylobacter jejuni* isolates studied by whole genome sequence.

Sample	Date of isolation	Location	ERIC-PCR cluster	MLST Sequence Type	Clonal Complex	<i>FlaA</i> svr
A11	24/09/2020	Porto	4	22	22	161
B3	23/10/2020	Monção	1	48	48	32;102
B9	23/10/2020	Monção	2	8569	179	571
B19	6/11/2020	Monção	2	148	21	36;36
Br2/1	20/11/2020	Monção	3	6461	353	67
Br4/2	20/11/2020	Monção	4	22	22	161
Br7/2	20/11/2020	Monção	3	6461	353	49;395
Br8/1	20/11/2020	Monção	3	6461	353	49
Br8/2	20/11/2020	Monção	4	22	22	161
Br9	20/11/2020	Monção	4	22	22	161

Regarding the various STs identified, a genomic-based epidemiological analysis was performed using the Bacterial Isolate Genome Sequence Database (BIGSdb) from pubMLST (<https://pubmlst.org/>).

From that database, ST-22 is distributed worldwide, including an isolate identified in Portugal in 2012. Often identified in humans, this ST is associated with numerous cases of Guillain-Barré syndrome and some cases of bacteremia. Strains have been isolated (although not in large numbers) from all types of livestock as well as cow's milk and environmental waters. In terms of companion animals, there are ten reported isolates (nine in dogs and one in a cat), all in Europe (Switzerland-8; UK-1 and Germany-1).

ST-6461 is a recently identified ST but, although its first isolate was reported only in 2012, it has been highly reported in the most recent years, especially in 2018 (date of the last report). In humans, only cases of gastroenteritis without further complications are associated, with cases only identified in Europe. Other sources of isolates include chickens (n=13) and lamb (n=1). No isolates belonging to dogs are found in this database.

With a great diversity of identified sources, ST-48 has already been isolated in a wide variety of animals (poultry, cattle, goats, lambs, etc.), animal foodstuffs (beef, chicken meat and dairy products) and environment (farms, water and beach sand). In the case of dogs, there are 17 identified isolates, all in Switzerland. Associated with a case of Miller Fisher Syndrome and two cases of bacteremia, this ST has been reported in humans all over the world.

There are only 66 isolates in this database belonging to the ST-148. With a single case reported in dogs (Switzerland), chickens were also identified as a source, with 23 isolates in Europe (Norway, Sweden, and Spain). Cases of human infection have been reported in Europe, Canada, and the USA and in one case, it was associated with Guillain-Barré syndrome (Netherlands).

Finally, ST-8569 is only associated with dogs, with two isolates in the database identified in France so far.

The genomes of the sequenced strains from this study were analyzed simultaneously with those of 50 strains isolated from humans during 2020. The crossing of data between both sources allowed the identification of isolates with high genetic proximity regarding ST and *flaA* svr; comparative studies between them were then carried out. Among the isolates from humans, one was identified as belonging to ST-6461, the same ST to which the isolates of cluster 3 belonged. Regarding ST-22, the one associated with cluster 4, and as so the most frequently found in this study, four isolates from humans were also identified as belonging to this ST.

ERIC-PCR of human and dog isolates belonging to the same STs (ST-22 and ST-6461) was then performed, in an attempt to confirm the ability of this technique to distinguish different isolates from *C. jejuni*. As seen in the Figure 4, regardless of the source, isolates from the same cluster of profiles in ERIC, belonged to the same ST. The analysis of eMLST (which increases the number of compared loci from 7 (MLST) to a total of 21) (Table A1 of the annex), allowed the detection of differences between isolates from the same ST. These differences were reflected in the ERIC profile, as for example in the case of human isolate 4093, where the ERIC profile had one band less compared to most of those belonging to the same cluster and whose eMLST showed differences in two loci. Finally, the discriminatory power of this technique was also tested with isolates with the same CC but different STs (data not shown), even with a more remote genotypic proximity, ERIC presented a good ability to cluster the isolates according to the respective CC. These results demonstrate a good correlation between ERIC-PCR and MLST.

After realizing that all isolates not identified by the multiplex PCR belonged to the same ST (ST-22), the genomes of the sequenced ST-22 isolates were analyzed to assess the presence and diversity of the PCR-multiplex target, the *cj0414* gene of the target of this

PCR. In fact, several mismatches were detected in the primer annealing sequence, that would prevent the primer annealing and subsequent polymerase extension, while there was a perfect correlation of primer/target sequence among ST6461 (data not shown).

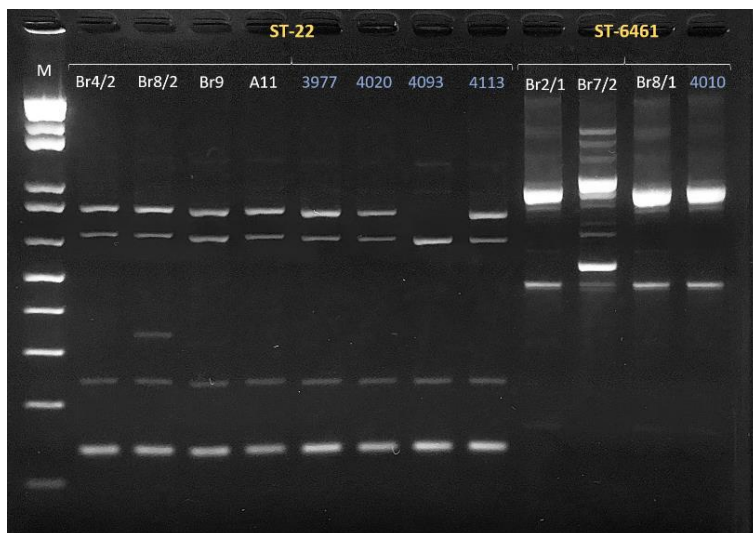


Figure 4: ERIC-PCR fingerprints of *Campylobacter jejuni* ST-22 and ST-6461 in a 2% agarose gel. Lane M, 1Kbp Plus ladder (Invitrogen). The ID of the sample is presented in white in the case of the dog isolates and in blue in the human isolates.

Finally, a comparative gene-by-gene analysis was performed to assess the genetic relationship between Portuguese (PT) ST-22 and ST-6461 isolates and other available genomes, also sharing the same *flaA* *svr* type (a total of 91 available genomes for ST6461, and 83 for ST22). A minimum spanning tree (MST) of the results regarding each ST was then generated.

From this analysis it was possible to observe that, regarding ST-6461 (Figure 5), not only all three dog isolates (Br2/1; Br7/2 and Br8/1) had the same wgMLST profile, but there was also a great proximity between these isolates and the human isolate 4010. This similarity is evidenced by the smaller distance between these two allelic profiles compared to all the others.

Even more relevant results were observed regarding ST-22 where, as seen in Figure 6, not only two isolates from dogs (Br4/2 and Br9) belonged to the same allelic profile as the human isolate 4020, but also all PT isolates but one (human isolate 4093) had very little allelic differences and formed a small cluster. When comparing with the remaining available isolates (Figure 7), it is evident how the cluster formed by the majority of PT isolates separates itself from the closest ones. It is also clear in this analysis, how isolate 4093 is distant from the others obtained in this study, belonging to a completely different cluster.

All these results reveal a very high phylogenetic proximity between dog and human isolates, an important fact to be considered.

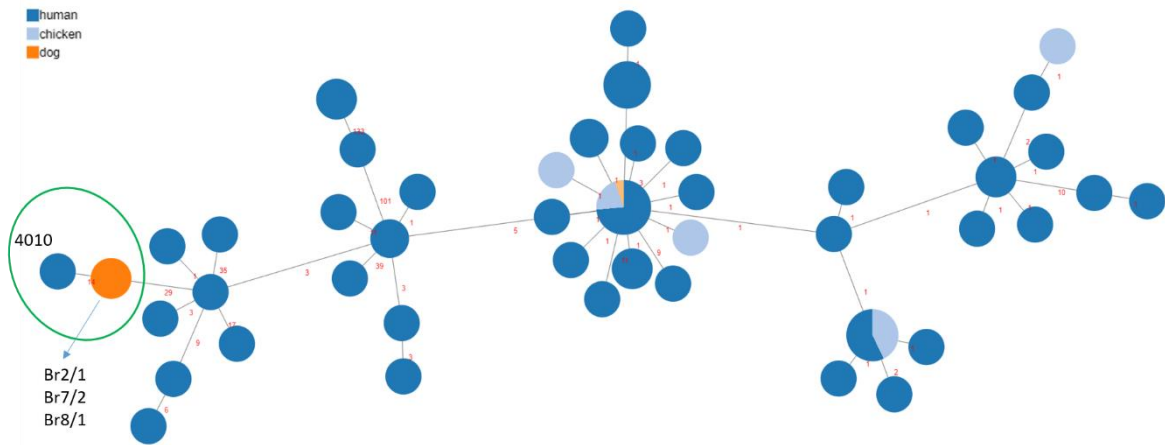


Figure 5: Phylogeny of *Campylobacter jejuni* ST6461 strains based on a dynamic gene-by-gene approach using a wgMLST schema with 2795 loci. The Minimum spanning tree was constructed using the goeBURST algorithm implemented in the PHYLOViZ online platform. Filled circles represent unique allelic profiles. The green circle represents the strains obtained in Portugal, 2020. The numbers in red on the connecting lines represent the allele differences (AD) between strains; only AD ≥ 5 are shown.

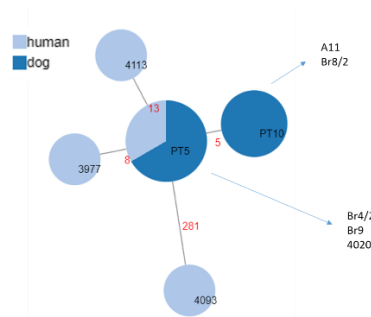


Figure 6: Phylogeny of *Campylobacter jejuni* ST-22 PT strains obtained based on a dynamic gene-by-gene approach using a wgMLST schema with 2795 loci. The Minimum spanning tree was constructed using the goeBURST algorithm implemented in the PHYLOViZ online platform. The numbers in red on the connecting lines represent the allele differences (AD) between strains; only AD ≥ 5 are shown.

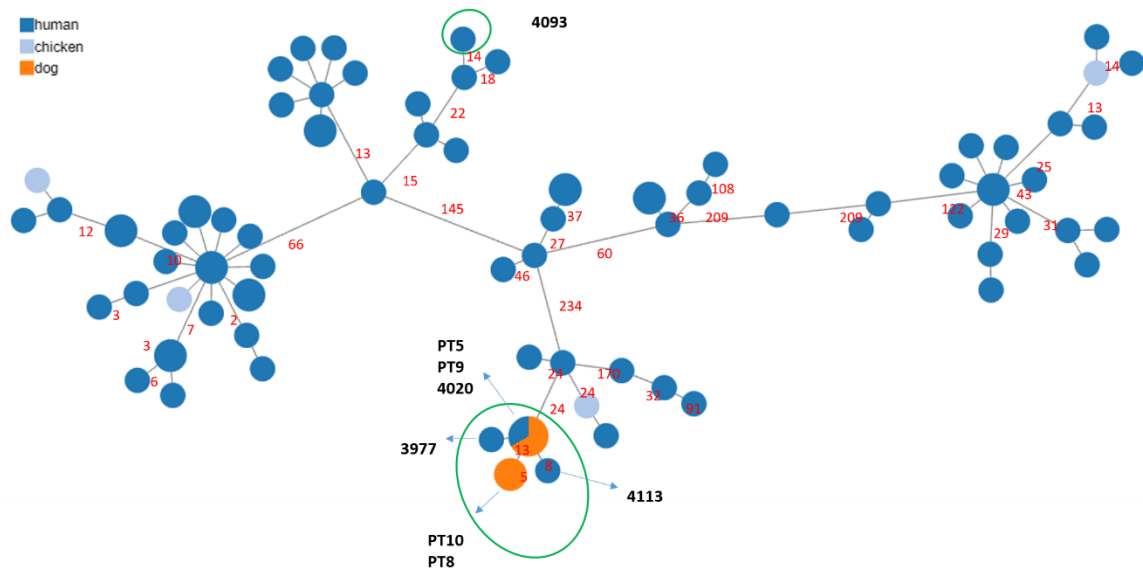


Figure 7: Phylogeny of *Campylobacter jejuni* ST22 strains based on a dynamic gene-by-gene approach using a wgMLST schema with 2477 loci. The Minimum spanning tree was constructed using the goeBURST algorithm implemented in the PHYLOViZ online platform. Filled circles represent unique allelic profiles. The green circle represents the strains obtained in Portugal, 2020. The numbers in red on the connecting lines represent the allele differences (AD) between strains; only AD ≥ 5 are shown.

4.2 – *CAMPYLOBACTER COLI*

The only *Campylobacter coli* isolate was obtained from a dog from group C, and presented resistance to CIP, TE and AMP; since it was a single isolate from this species, it will be included in another study regarding *C. coli* from different sources.

4.3 – *CAMPYLOBACTER UPSALIENSIS*

A total of four isolates of *Campylobacter upsaliensis* was obtained in this study. Together with these isolates, two isolates from humans (4048 and 4182) and one from river water (W36-2), obtained in Portugal in 2020, were also analyzed. The results of antimicrobial susceptibility testing are illustrated in Figures 8 and 9 and reveal similar profiles of antibiotic susceptibility, with resistance to CIP, TE and AMP being found in samples from all three sources.

Regarding the ERIC-PCR (Figure 10), differences in the profiles obtained with this technique show a variability between isolates from different sources, but also a cluster of isolates with similar profiles. As can be seen in Figure 10, the profiles of A10 and A45, two dog isolates from Oporto, resemble the profiles of W36-2 (river water) and of 4182 (human isolate). This similarity may mean greater genomic proximity between these isolates, although a validation of the results is necessary. The other two isolates from dogs, one from Oporto (A5) and one from Monção (C7) did not resemble each other or any other profile, the same was observed in the isolate 4048, the second isolate from humans. Altogether, these results showed a good potential for the use of ERIC in typing *C. upsaliensis*, as a complement of other molecular markers.

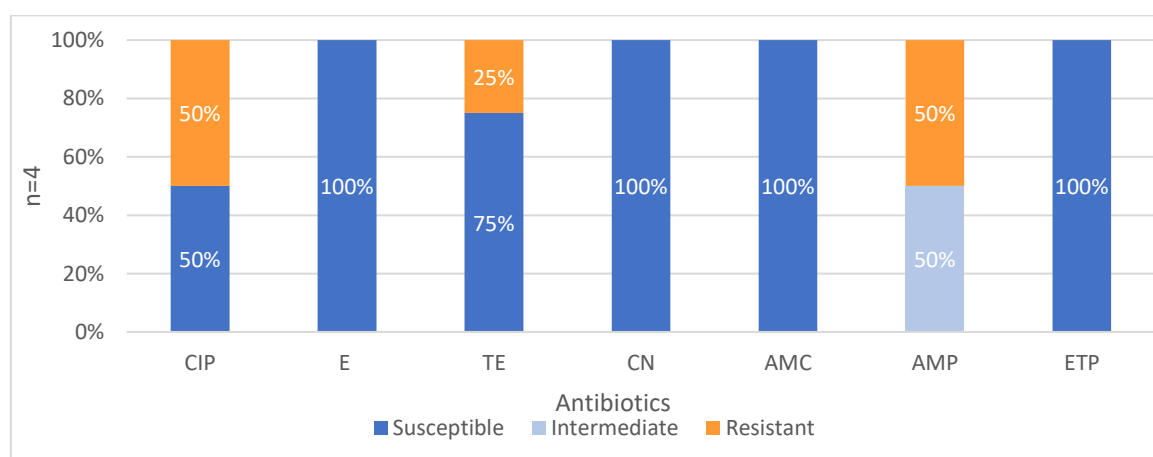


Figure 8: Antibiotic susceptibility (%) of *Campylobacter upsaliensis* isolates from dogs (n=4).

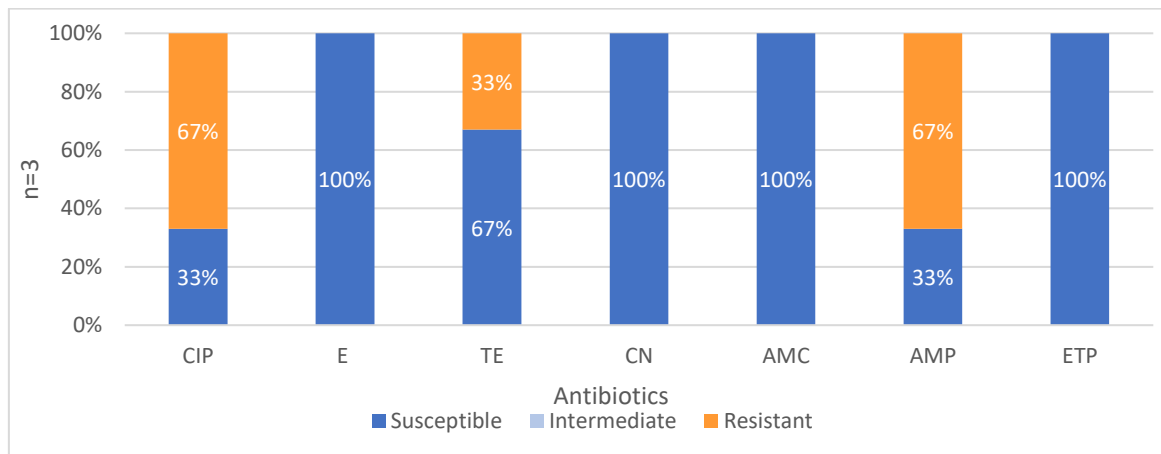


Figure 9: Antibiotic susceptibility (%) of *Campylobacter upsaliensis* isolates from humans and river water (n=3).

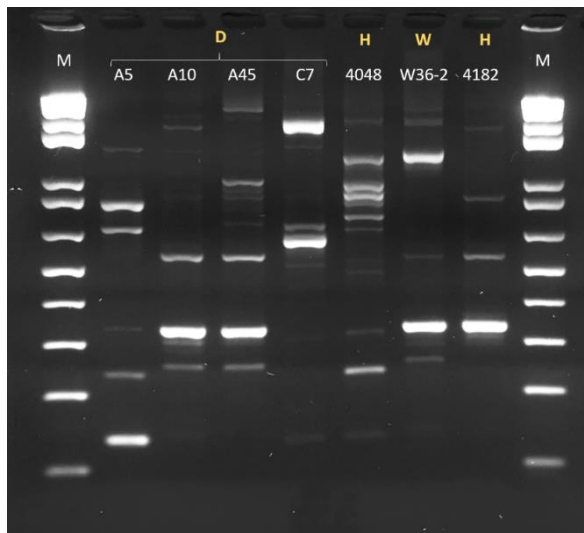


Figure 10: ERIC-PCR fingerprints of *Campylobacter upsaliensis* isolates in a 2% agarose gel. Lanes M, 1Kbp Plus ladder (Invitrogen). The yellow letters represent the origin of the sample (D-dog; H-human and W-water).

4.4 – *CAMPYLOBACTER LARI*

Only nine dogs from group B carried *Campylobacter lari*. The PCR Multiplex was only able to identify them as *Campylobacter* spp. and species identification was obtained by MALDI-TOF. Since all isolates belonged to the same environment, some of them from the same animals, and since no relevant antimicrobial resistance is described regarding *C. lari*, the susceptibility testing was not performed. These were characterized by ERIC-PCR, in order to compare the profiles of the samples. As seen in figure 11, the results from ERIC show that they all had very similar profiles, forming one only cluster. The only difference observed was regarding the profile from isolate B7, which had one band less compared to the others. This is a curious finding since it refers to the isolate obtained in the first sample of dog B7 (boarhound) and whose isolate from the second sample (Br7/1), had a profile more identical to the others.

Although a variety of profiles has not been obtained to assess the ability of this technique to distinguish different types of *C. lari*, the fact that the observed profile had a considerable number of bands (which allows a more accurate comparison), reveals that it is worthy to further assess the usefulness of this technique regarding this species.

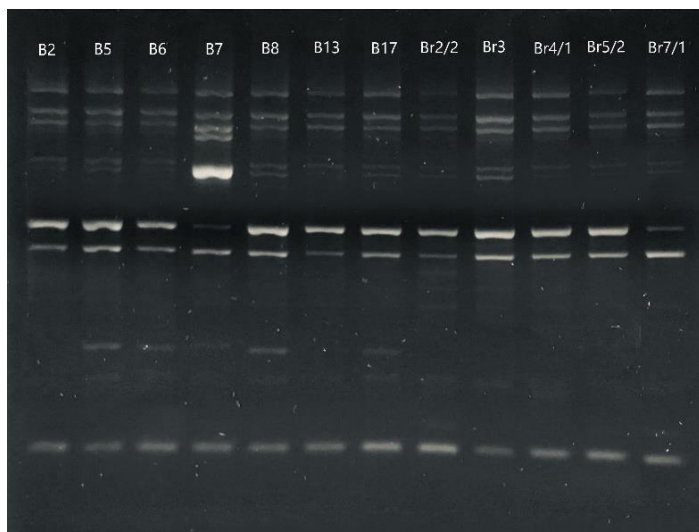


Figure 11: ERIC-PCR fingerprints of *Campylobacter lari* isolates from boarhounds in a 2% agarose gel.

5 – DISCUSSION

All *Campylobacter* species found in this study were previously reported in dogs (Giacomelli et al., 2015; Iannino et al., 2019; Olsson Engvall et al., 2003). Although (due to the bias present in the study) assumptions cannot be made about prevalence differences in the two geographic areas (Oporto, group A, and Monção, groups B and C), with all dogs carrying *Campylobacter* spp. in group A presenting diarrhea, and none from groups B and C presenting it, the disparity in terms of association of clinical signs with the presence of the bacterium, was quite evident. Not much speculation can be made about why this happened, even though they belonged to quite different environments. It would be of interest to further investigate whether this divergence is due to differences in terms of the host, or the environment. Between the samples from the two clinics (groups A and C), given the small number of isolates obtained, it is not possible to calculate risk factors between different ages, genders, or the reason for the visit to the hospital.

Regarding the detection of mixed colonization in group B, it is possible that considering that the second samples were obtained at a more advanced period of the hunting season, when the animals had already been exposed to a greater diversity of environments and animals, justifies this observation. However, it cannot be ignored that, over time, an improvement in the technique of isolation and identification of the samples

can also be expected, a fact that may have facilitated the distinction of different colonies in the same culture.

Besides being the only species detected in all three groups, *C. jejuni* was the most isolated species in this study. These results are relevant from a public health point of view, since *C. jejuni* is the leading responsible for human infections (EFSA & ECDC, 2020).

Regarding antimicrobial resistance, previous studies in dogs have reported resistance to CIP (9-58%), TE (12-32%) and to E in a lesser extent (0-12%) (Acke et al., 2009; Lee et al., 2004; Rossi et al., 2008; Sahin et al., 2014). Although no resistance to E was observed in this study, which, in Portugal, is much relevant in *C. coli*, higher resistance rates to CIP and TE were found, and the results are very similar to those recently reported by EFSA regarding *C. jejuni* from humans in Portugal, where rates of 94% resistance to ciprofloxacin and 79% to tetracyclines were described (EFSA & ECDC, 2020). In fact, in Portugal, as is the case worldwide, the biggest problems are related to the resistance levels observed in broilers and turkeys, reaching above 90% for both CIP and TE, resulting in human contamination with highly resistant strains (EFSA & ECDC, 2020).

When analyzing the isolates from boarhounds, as it was possible to confirm with phenotypic (antimicrobial susceptibility) and genotypic (ERIC-PCR and WGS) assessment, there was a high diversity of *C. jejuni* strains between animals and between sampling events. From the first samples collected, two isolates belonging to different STs (ST-48 and ST-8569) were obtained. None of the sequenced isolates from the second sampling point belonged to these same STs, as three of them were ST-22 and another three were ST-6461. This is an interesting finding since all dogs lived in the same household, under the same conditions. This could possibly mean that being a boarhound, with all that that entails (close contact with the environment, wild species, and other packs), could be a bigger risk factor for carrying *C. jejuni* than living in a kennel. For this to be confirmed, further studies should be done, not only including more sampling events over time, but also covering other boarhound packs.

Also curious was the fact that a dog from Oporto (A11), a “city dog”, had the same wgMLST allelic profile as a boarhound (Br8/2), more than 100 kilometers away. This indicates not only a wide geographic distribution (rural and urban) but also a prevalence among dogs with different purposes (companionship and hunting).

Among the five different STs identified in this study, four of them (ST-22; ST-48; ST-148 and ST-6461) represented STs widely found in humans, revealing a surprisingly close epidemiological relationship between the isolates present in both species. This relationship becomes even more relevant when looking at the MSTs, where the PT isolates from both STs (ST-22 and ST-6461) formed high proximity clusters, reinforcing the need for epidemiological studies regarding dogs as a possible source of human infection. This

consideration could be of particular importance with some STs, like in the case of ST-22. This particular ST, the most common among dogs in this study, has been highly associated with chronic complications such as Guillain-Barré syndrome, being overrepresented amongst patients with this post-infection complication (Islam et al., 2009; Revez et al., 2011; Taboada et al., 2007). Given that, compared to others, this ST is not as frequently found among most common animal sources (poultry, pigs, etc.), and given the close relationship between the isolates obtained in this study, dogs could be a more relevant source than currently considered (De Haan et al., 2010; Dingle et al., 2002; Revez et al., 2011).

As the second main responsible for human campylobacteriosis, ubiquitous *C. coli* is often isolated in poultry, with a prevalence sometimes higher than *C. jejuni* (EFSA & ECDC, 2020). The fact that this is the *Campylobacter* species with which more AMR problems are associated, raises the importance of monitoring its prevalence and resistance levels (EFSA & ECDC, 2020). In dogs, *C. coli* is reportedly less frequent comparing to *C. jejuni* and *C. upsaliensis* (Iannino et al., 2019), this fact is in the line with the results from this study, where only one isolate was obtained.

Despite the unquestionable relevance that *C. jejuni* and *C. coli* have in campylobacteriosis, the role that other species of *Campylobacter* play in the etiology of human gastroenteritis has been increasingly questioned (Costa & Iraola, 2019; Man, 2011). Since the classic diagnostic methods have been optimized aiming the two most common species, they are often not suitable for the isolation of more fastidious ones, which are frequently susceptible to the antibiotics present in the selective media and may need different temperatures or times of incubation (Bourke et al., 1998; Man, 2011).

This could also be a possible explanation for the number of *C. upsaliensis* isolates in this study being lower than the usually reported; as this species is generally the most common in dogs (Carbonero et al., 2012; Iannino et al., 2019). With dogs and cats as its main reservoir, *C. upsaliensis* has increasingly been associated with human infections. Although usually associated with enteritis, this bacterium has reportedly caused abortion, sepsis, hemolytic-uremic syndrome and Guillain-Barré syndrome (Carter & Cimolai, 1996; Gurgan & Diker, 1994; Ho et al., 1997; Nakamura et al., 2015; Rowe & Madden, 2014). Isolation of *C. upsaliensis* from a breast abscess and a from hepatic cyst were also reported (Gaudreau & Lamothe, 1992; Ohkoshi et al., 2020). In dogs, as generally with *Campylobacter* spp., this species is more commonly seen as commensal, with a prevalence of around 20% in healthy dogs, despite that, it has been associated with enteritis as well as with post-infection complication like polyradiculoneuritis (Chaban et al., 2010; Iannino et al., 2019; Martinez-Anton et al., 2018). In terms of antimicrobial resistance in humans, the few studies regarding it, report resistance rates of approximately 6% to CIP and around 12 % to E (Goossens et al., 1990; Vandenberg et al., 2006). Regarding dogs, only resistance to

CIP and AMP has been reported, with rates of approximately 8% in both cases (Olkkola et al., 2015; Rossi et al., 2008). Different results were obtained in this study, where although no resistance to E was found, higher rates of resistance to CIP (50% in dogs and 67% in humans and water) and AMP were observed (25% in dogs and 33% in humans and water), along with two isolates (one from a dog and another one from a human) presenting resistance to TE, something not previously reported.

Despite the lack of validation regarding the ERIC-PCR in this species, the presence of similar profiles between different sources, combined with the similarities regarding antibiotic resistance, reinforces the often overlooked zoonotic potential that this species of *Campylobacter* has, as well as the role that dogs can play in its transmission.

Regarding *C. lari*, despite the high number of isolates found in this study, they were all obtained from the same group of dogs, the majority from the same animals sampled twice. As no positive control was used when performing the multiplex PCR and no *C. lari* isolate was sequenced, it is not possible to know if the fact that this technique could not identified these isolates was due to a genomic variation or to some technical problem regarding the reaction. The fact that the results from ERIC-PCR, seem to indicate identical genotypes, coupled by the fact that a 5-month-old female dog, born in that kennel and that had never left it, presented the bacterium, makes it plausible to assume that it belongs to the commensal flora present in that pack.

Originally isolated from gulls, *C. lari* is usually associated with marine environments and can be isolated from aquatic birds and several kinds of shellfish (Costa & Iraola, 2019; Rowe & Madden, 2014; Skirrow & Benjamin, 1980). Despite that fact, isolation from livestock and dogs, although with a lower prevalence, is also described (Aarestrup et al., 1997; Chaban et al., 2010; Scanlon et al., 2013). Regarding dogs, not only the prevalence reported is very low (1-2%), but also no especial complications associated with this infection are reported (Iannino et al., 2019). Associated with human enteritis, cases of bacteremia and post-infection complications like reactive arthritis, have also been reported (Broczyk et al., 1987; Krause et al., 2002; Rowe & Madden, 2014; Werno et al., 2002).

In conclusion, despite being a first insight to *Campylobacter* spp. in dogs in Portugal, this study revealed a surprising proximity between the *C. jejuni* isolates obtained in dogs and those isolated from humans. Also, regarding *C. upsaliensis*, despite the clear need of further study and validation of the results obtained, there is an apparent similarity between dogs and humans isolates that should not be overlooked. Thus, seen more and more as our faithful companions and members of our families, the role that dogs can play in the transmission of *Campylobacter* cannot be ignored and further studies should be done to assess the real impact that this relationship may have.

In terms of the veterinary clinical relevance, although the number of isolates obtained was not very high, the apparent relationship between the bacteria and gastrointestinal symptoms in dogs from an urban region (Oporto), deserves further study. Also, clinicians should pay greater attention to this organism since, in general, even when it is considered, appropriate sample collection and processing are not carried out, making its identification very unlikely.

Extensive epidemiological studies are the foundation of a "One Health" approach. For that to happen, an appropriate selection of typing techniques is essential, and harmonization and standardization of typing methods and database is necessary for an efficient global sharing of information. Regarding ERIC-PCR, although an optimization that allows obtaining more complex fingerprints (thus allowing a more discriminatory comparison), and a validation with a greater number of isolates are still needed, this seems to be a very suitable option for routine genotyping analysis. Given its effortlessness and low cost, this technique may be of special interest regarding sources of *Campylobacter* not normally studied (as it is the case with dogs), as well as regarding the study of non-*coli*/non-*jejuni* *Campylobacter* species, where usually only a phenotypic evaluation through antimicrobial susceptibility is performed.

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ANNEX

Table A1: eMLST and *flaA* svr of *Campylobacter jejuni* ST-22 and ST-6461 isolates

	ST-22								ST-6461			
	Br4/2	Br8/2	Br9	A11/2	3977	4020	4093	4113	Br2/81	Br7/2	Br8/1	4010
<i>aspA</i>	1	1	1	1	1	1	1	1	2	2	2	2
<i>fadk</i>	17	17	17	17	17	17	17	17	28	-	28	28
<i>fasd</i>	12	12	12	12	12	12	12	12	4	4	4	4
<i>fdapE</i>	92	92	92	92	92	92	92	92	105	105	105	105
<i>fdlA</i>	16	16	16	16	16	16	16	16	11	11	11	11
<i>feftS</i>	26	26	26	26	26	26	26	26	1	1	1	1
<i>ffumC</i>	11	11	11	11	11	11	11	11	6	6	6	6
<i>filvD</i>	14	14	14	14	14	14	69	14	18	18	18	18
<i>flepP</i>	8	8	8	8	8	8	8	8	5	5	5	5
<i>fmdh</i>	20	20	20	20	20	20	20	20	4	4	4	4
<i>fmutY</i>	92	92	92	92	92	92	64	92	1	1	1	1
<i>fnuoH</i>	15	15	15	15	15	15	15	15	1	1	1	1
<i>fpgi</i>	2	2	2	2	2	2	2	2	7	7	7	7
<i>ftpi</i>	1	1	1	1	1	1	1	1	12	12	12	12
<i>fyphC</i>	15	15	15	15	15	15	15	15	1	1	1	1
<i>glnA</i>	3	3	3	3	3	3	3	3	17	17	17	17
<i>gltA</i>	6	6	6	6	6	6	6	6	5	5	5	5
<i>glyA</i>	4	4	4	4	4	4	4	4	2	2	2	2
<i>pgm</i>	3	3	3	3	3	3	3	3	10	10	10	10
<i>tkk</i>	3	3	3	3	3	3	3	3	59	59	59	59
<i>uncA</i>	3	3	3	3	3	3	3	3	6	6	6	6
										49		
<i>FlaA svr</i>	161	161	161	161	161	161	106	296	67	395	49	67

Note: The ID of the sample is presented in grey in the case of the dog isolates and in blue in the human isolates