UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

EXTENDED–SPECTRUM–BETA-LACTAMASES, CEPHALOSPORINASES AND CARBAPENEMASE-PRODUCING *Escherichia coli* IN THE HUMAN-DOG INTERFACE

ADRIANA DE JESUS INÁCIO BELAS

Orientador(es): Professora Doutora Maria Constança Matias Ferreira Pomba

Professor Doutor João André Nogueira Custódio Carriço

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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Faculdade de Medicina Veterinária da Universidade de Lisboa, 17 de março de 2021

Assinatura: 

To my daughter and husband.

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Abstract

Extended–spectrum–beta-lactamases, cephalosporinases and carbapenemase-producing *Escherichia coli* in the human-dog interface.

Extended–spectrum–beta-lactamases (ESBLs), cephalosporinases (encoded by the ESBLs and Ampc genes, respectively) and carbapenemase–producing *Escherichia coli* have become a major public health concern to both human and animal health. Urinary tract infections (UTI) are one of the most frequent bacterial infections in both human and companion animals. Uropathogenic *E. coli* (UPEC), belonging to extraintestinal pathogenic *E. coli* (ExPEC), is the most common bacterium isolated from companion animals. Moreover, the close contact of companion animals with humans creates opportunities for interspecies transmission of resistant bacteria and genes.

E. coli from companion animals with UTI were found to harbour important antimicrobial resistance mechanisms and to belong to high-risk human clonal lineages, namely third-generation cephalosporin (3GC)-resistant *E. coli* O25b:H4-B2-ST131-*H*30/*H*30Rx, CC23 and ST648. In this work *E. coli* O25b:H4-ST131-*H*30/*H*30Rx was described for the first time in Europe in companion animals. Furthermore, the bla_{CMY-2} producing *E. coli* ST648 is the most common high-risk clonal lineage causing UTI in companion animals from the Lisbon area. Companion animals also seem to be reservoirs of bacteria and clinically important resistance genes, such β-lactams genes (classe A and C) which supports their role as reservoirs.

The detection of faecal high-risk clone OXA-181-producing- *E. coli* ST410 strains that were closely related to uropathogenic clinical human strains was also an important finding and to our best knowledge was the first description in Portugal and Europe.

These studies highligth the importance of companion animals as reservoirs of pathogenic *E. coli* harbouring important antimicrobial resistant genes. The emergence and spread of multidrug-resistant (MDR) *E. coli* in the natural environment by companion animal faecal contamination is also a concern towards animal and human health. These results point to need for control measures to prevent the dissemination of MDR ESBLs/AmpC and carbapenemases – producing bacteria from companion animals.

Keyword: *Escherichia coli*; high-risk clones; Enterobacteriaceae; genes; companion animals.

Resumo

Escherichia coli produtora de Beta-lactamases de Espectro Alargado e Carbapenemases na interface Homem-cão.

A *Escherichia coli* produtora de beta-lactamases de espectro alargado (ESBLs / Ampc) e de carbapenemase tornou-se uma grande preocupação de saúde pública em termos de saúde humana e animal. As infeções do trato urinário (ITU) são uma das infeções bacterianas mais frequentes nos humanos e nos animais de companhia. A *E. coli* uropatogénica pertencente à família da *E. coli* patogénica extra-intestinal é a bactéria mais comum isolada em animais de companhia. Além disso, o contato próximo dos animais de companhia com os seres humanos permite oportunidades para a transmissão de bactérias resistentes e genes entre as espécies.

Descobriu-se que *E. coli* de animais de companhia com ITU possuiem importantes mecanismos de resistência antimicrobiana e pertencem a linhagens clonais humanas de alto risco, nomeadamente, *E. coli* resistente as cefalosporinas de terceira geração (3GC) O25b: H4-B2-ST131-*H*30 / *H*30Rx, CC23 e ST648. Neste trabalho, a *E. coli* O25b: H4-ST131-*H*30 / *H*30Rx foi descrita pela primeira vez na Europa em animais de companhia. Além disso, a *E. coli* ST648 produtora de bla_{CMY-2} é a linhagem clonal de alto risco mais comum que causa ITU em animais de companhia na área de Lisboa.

Os animais de companhia podem ser também potenciais reservatórios de bactérias e de genes de resistência clinicamente importantes, como os genes das β-lactamases (classes A e C).

A detecção de estirpes fecais de linhagens clonais de alto risco *E. coli* produtora de OXA-181 ST410, relacionadas com estirpes clínicas uropatógenicas humanas foi também um achado importante e para nosso conhecimento foi a primeira descrição em Portugal e na Europa.

Estes estudos destacam a importância dos animais de companhia como reservatórios de *E. coli* patogénica que contém importantes genes de resistência a antimicrobianos. O aparecimento e disseminação de *E. coli* multirresistente (MDR) no ambiente natural por contaminação fecal de animais de companhia é também uma preocupação para a saúde humana e animal. Estes resultados apontam para a necessidade de medidas de controlo para prevenir a disseminação de bactérias produtoras de ESBLs / AmpC e carbapenemases por animais de companhia.

Palavra-chave: *Escherichia coli*; linhagens clonais de alto risco; Enterobacteriaceae; genes; animais de companhia.

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Abbreviations

AACs	Aminoglycoside N-acetyltransferases
Ag43	Antigen 43
AMEs	Aminoglycoside-modifying enzymes
AmpC	Cephalosporinases
AMRs	Antimicrobial resistance genes
ANTs	Aminoglycoside O-nucleotidyltransferases
APEC	Avian pathogenic *E. coli*
APHs	Aminoglycoside O-phosphotransferases
AT	Autotransporter
BLIs	β-lactamase inhibitors
CATs	Chloramphenicol acetyltransferases
CDTs	Cytolethal distending toxins
cgs	Curlin subunit gene
CIA	Critically important antimicrobial
DAF	Decay-accelerationg factor
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
EAEC	Enteroaggregative *E. coli*
EARS-Net	European Antimicrobial Resistance Surveillance Network
ECOR	*E. coli* reference collection
ecp	*E. coli* commom pilus
EDTA	Ethylenediaminotetraacetic acid
EIEC	Enteroinvasive *E. coli*
ESBLs	Extended spectrum β-lactamases
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
ExPEC	Extraintestinal pathogenic *E. coli*
HIA	Highly important antimicrobial
HLGR	High-level gentamicin resistance
HP-CIA	Highest priority critical important antimicrobial
HPI	High-pathogenicity Island
IA	Important antimicrobial
IMP	Imipenemase
ISs	Insertion sequences
LPS	Lipopolysaccharide

mar	Multiple antibiotic resistance
MBL	Metallo-β-lactamases
MDR	Multidrug resistant
MICs	Minimal Inhibitory concentrations
MLEE	Multi locus enzyme electrophoresis
MLST	Multi locus sequence typing
MR-CoNS	Methicillin-resistant coagulase-negative staphylococci
MRSA	Methicillin-resistant *staphylococcus aureus*
MRSI	Methicillin-resistant *staphylococcus intermedius*
MRSP	Methicillin-resistant *staphylococcus pseudintermedius*
NDM-1	New Delhi metallo-β-lactamase
ORFs	Open reading frames
PABA	Para-aminobenzoic acid
PAIs	Pathogenicity islands
PBP	Periplasmic binding protein
PBPs	Penicillin-binding proteins
PMQR	Plasmid mediated quinolone resistance
QRDR	Quinolone resistance determining region
RMTs	16S ribosomal RNA methyltransferases
RTI	Respiratory tract infections
SPATEs	Serine protéase AT proteins of Enterobacterales
SSTI	Skin and soft tissue infection
TLR4	Toll-like receptor 4
UP1a	Uroplakin 1A receptor
UPEC	Uropathogenic *E. coli*
usp	Uropathogenic specific protein
UTI	Urinary tract infection
Vat	Vacuolating autotransporter toxin
VFs	Virulence factors
VGs	Virulence genes
VIM	Verona integron-encoded metallo-β-lactamase
WHO	World Health Organization

List of publications and presentations

Book Chapers (available in this thesis annex 1 and 2):

Marques C., **Belas A.**, Pomba C. (2020). Antimicrobial Resistance Trends in Dogs and Cats with Urinary Tract Infection. In: Freitas Duarte A., Lopes da Costa L. (eds) Advances in Animal Health, Medicine and Production. Springer, Cham. https://doi.org/10.1007/978-3-030-61981-7_13.

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Chapter 1

Introduction

1.1 The antimicrobial use in veterinary medicine

Antimicrobial resistance represents a major risk to human health. As a result, we are faced with potential therapeutic failure, reduction of treatment options and severe disease outcome forcing physicians to use last resource antimicrobials such as carbapenems. It is known that the use of antimicrobials will increase the risk of antimicrobial resistance and the risk of colonization by non-commensal bacteria and infection by foodborne pathogens (Barza and Travers 2002). There is a growing concern related to the use of antimicrobials in food-producing and companion animals as a potential source for antimicrobial resistance in humans (Greko et al. 2009; Catry et al. 2010; van Duijkeren et al. 2014; Pomba et al. 2017). For some antimicrobial's resistance prevalence in animals and humans is still increasing or remains unchanged at a substantial level (WHO-AGISAR 2009). A unique aspect related to antimicrobial resistance in companion animals is their close contact with humans providing opportunities for interspecies transmission of resistant bacteria. Use of antimicrobials that are critically important for human health in companion animals is an additional risk factor for emergence and transmission of antimicrobial resistance. Public health risks associated with transfer of antimicrobial resistance from companion animals are reviewed in The European Medicine Agency and its Antimicrobial Working Party reflection paper warning of existence of antimicrobial resistance microbiological hazards coming from companion animals to humans (EMA 2015). Of special concern is the situation in which the use of antimicrobials in companion animals contributes to resistance against last resort antimicrobials used in human medicine. Problems of resistance development and of infection control in companion animal hospitals are mimicking those in human hospitals (Morley 2004; ECDC et al. 2009).

Since 2010, the European Medicine Agency started reporting data on antimicrobial sales for companion animals. Moreover, the European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) project collects information how antimicrobials are used in companion animals across the European Union by the implementation of the interactive ESVAC database that complements the annual ESVAC

report (ESVAC 2020). This type of information is important to identify possible risk factors that could lead to the development and spread of antimicrobial resistance (ESVAC 2020).

In Portugal, β-lactams, such as penicillins, are the most prescribed antimicrobials followed by macrolides, lincosamides, streptogramins, quinolones, tetracyclines and sulfonamides-trimethoprim (ECDC 2018). In Veterinary medicine, beta-lactams are also the most commonly prescribed antimicrobials in companion animals, especially, amoxicillin and amoxicillin–clavulanate. Furthermore, lincosamides, quinolones, macrolides, tetracyclines (doxycycline), nitroimidazoles and sulfonamides-trimethoprim are also used in small animal practice (EMA 2018, EMA 2019). In Figure 1 is represented the proportion of the total sales of the different veterinary antimicrobial's classes, in mg/PCU, in the European countries for 2018, including Portugal (EMA 2020). However, antimicrobial sales are different between countries (Figure 1).

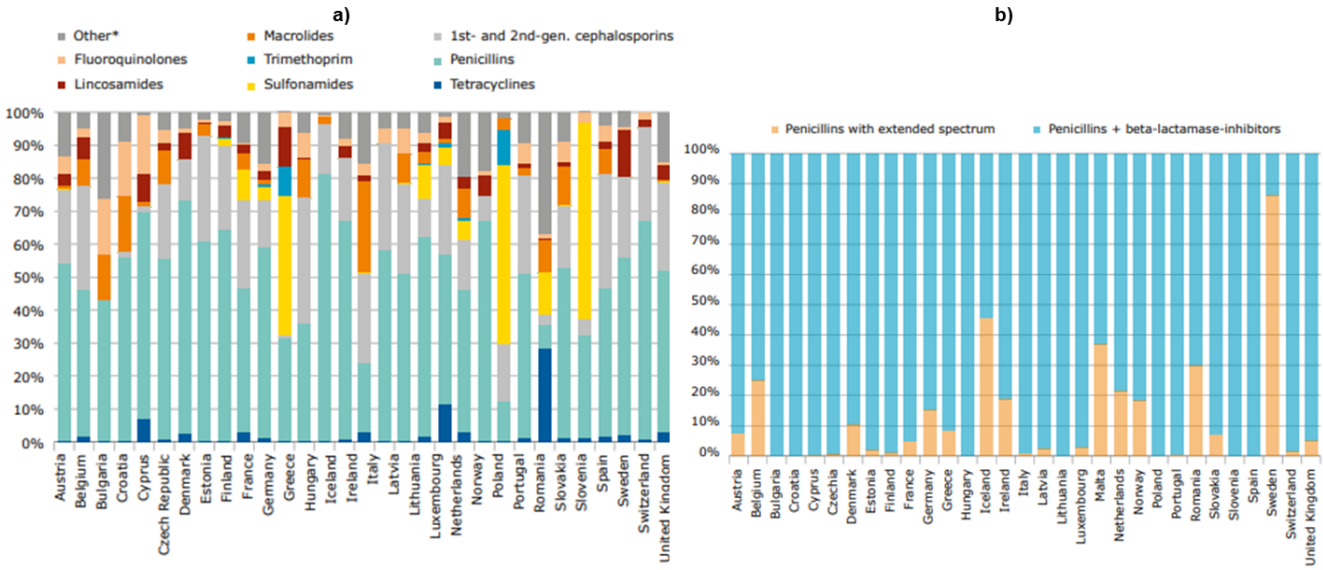


Figure 1: Veterinary antimicrobial class sales of antimicrobial tablets, in mg/PCU, in the 31 European countries, for 2018 (adapted from EMA 2020).

Legend: a) *Small amounts of aminoglycosides, amphenicols, nitrofuran derivatives, pleuromutilins, polymyxins and other antimicrobials were sold in some countries; b) distribuition of sales (by weight of active ingredient) of tablets containing penicillins by subclasses, by country, in 2018.

However, according to sales data (mg / PCU) from 25 countries reported to ESVAC for every year from 2011 to 2018 there was an reduction of 34.6% in sales (mg / PCU) (Figure 2) and a decrease in sales (in mg / PCU) of more than 5% for 18 of the 25 countries. During this same period, sales (mg / PCU) of 3rd and 4th generation cephalosporins decreased by 24.4%, while sales of fluoroquinolones decreased by 4.2% and sales of other quinolones by 74.4% (Figure 2) (EMA 2020).

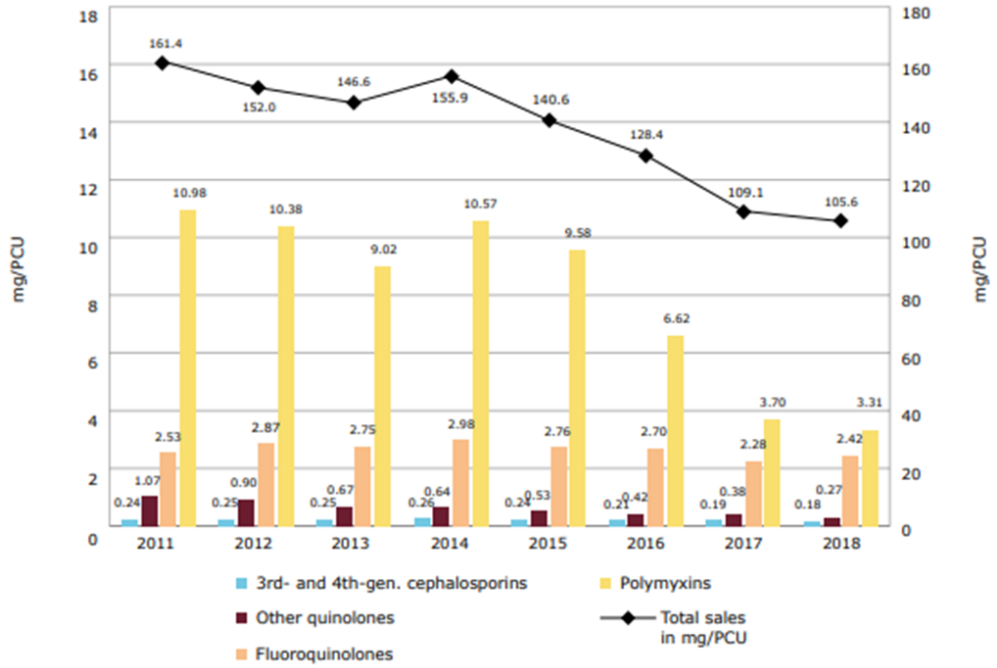


Figure 2: Changes in aggregated overall sales in mg/PCU, as well as sales of fluoroquinolones, other quinolones, 3rd and 4th-generation cephalosporins and polymyxins, for 25 EU/EEA countries*, from 2011 to 2018 (adapted from EMA 2020).

Legend: *Austria, Belgium, Bulgaria, Cyprus, Czechia, Denmark, Estonia, Finland, France, Germany, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Netherlands, Norway, Poland, Portugal, Slovakia, Slovenia, Spain, Sweden and the United Kingdom.

Urinary tract infections (UTI) and skin and soft tissue infections (SSTI) are the two most common infections in companion animals (Couto et al. 2016; Marques et al. 2018), and they account for substantial antimicrobial use. They are also increasingly associated with resistant pathogens, including species and strains that cause human diseases. The incidence of disease and similarity of human and animal pathogens is compounded by similar treatment trends in humans and companion animals. Beta-lactams (namely amoxicillin/clavulanic acid and third generation cephalosporins) and fluoroquinolones (namely second-generation fluoroquinolones) are the most commonly used antimicrobial classes in companion animals with UTI or SSTI (Hillier et al. 2014; Weese et al. 2019). Amoxicillin/clavulanic is classified by WHO as a critically important antimicrobial (CIA) and third generation cephalosporins and fluoroquinolones as highest priority critically important antimicrobials (HP-CIAs), respectively in human medicine (WHO 2019). This antimicrobial use, both non-prudent and indiscriminate, has led to the increasing trends of antimicrobial resistance and multidrug-resistant bacteria observed in companion animals with UTI and SSTI (Couto et al. 2016; Marques et al. 2018), as well as lead to increasing colonization of animals with multidrug resistant bacteria after successful clinical treatment (Beck et al. 2012).

It should be noted that the most sold antimicrobials in companion animals worldwide overlap those routinely used in human medicine and are considered as critically important antimicrobials to humans by the World Health Organization (WHO) (WHO 2017). Furthermore, in contrast to food-producing animals, the prescription of antimicrobials only approved for human use may occur under the cascade principles in companion animals (Pomba et al. 2017). This represents an additional antimicrobial resistance selective pressure towards last resorts antimicrobials and warrants the need for a One Health approach to fight the dissemination of antimicrobial resistance.

1.1.1 β-lactams resistance

β-lactams are bactericidal agents that prevent bacterial cell-wall synthesis by binding to an active serine site from penicillin-binding proteins (PBPs), which represents a class of enzymes with essencial roles in the synthesis of bacterial cell wall that leads to cellular death (Smet 2010; Bush and Bradford 2016; Eiamphungporn et al. 2018). However, β-lactams resistance can occur by multiple molecular mechanisms such as: 1) production of efflux pumps to expel β-lactams, 2) modification or reduced production of outer membrane porins to reduce the β-lactams, 3) alterations of PBPs, 4) production of β-lactamase for inactivating β-lactams (Jacoby et al. 1988; Jarlier et al. 1988; Eiamphungporn et al. 2018). Although, the major mechanism of resistance against β-lactams in Gram-negative bacteria is production of β-lactamases, which irreversibly open the β-lactam ring of antimicrobials (Eiamphungporn et al. 2018). One of the strategies to overcome β-lactamase-mediated resistance is the development of β-lactamase inhibitors (BLIs). These small molecule inhibitors were discovered and have been applied in combination with β-lactams for efficient therapy (Eiamphungporn et al. 2018). Clavulanic acid acts synergistically with penicillins and cephalosporins allowing the β -lactam to kill bacteria producing clavulanic-acid sensitive β –lactamases. Other β -lactamase inhibitors include sulbactam and tazobactam (Fernandes et al. 2013; Bush and Bradford 2016). In veterinary medicine the most used combination is amoxicillin/clavulanate. The Figure 3 shows the discovery of β-lactams and BLIs and the development of resistance over the years.

The β-lactamases enzymes are produced in the periplasmic space by Gram-negative bacteria and the genes for β-lactamases enzymes can be present on the bacterial chromosome and on mobile genetic elements (plasmids and transposons) (Eiamphungporn et al. 2018). The β-lactamase enzymes produced by bacteria are diverse and over the years some classification systems have been proposed: the Ambler classification, which is based on amino acid sequences similarities - protein homology

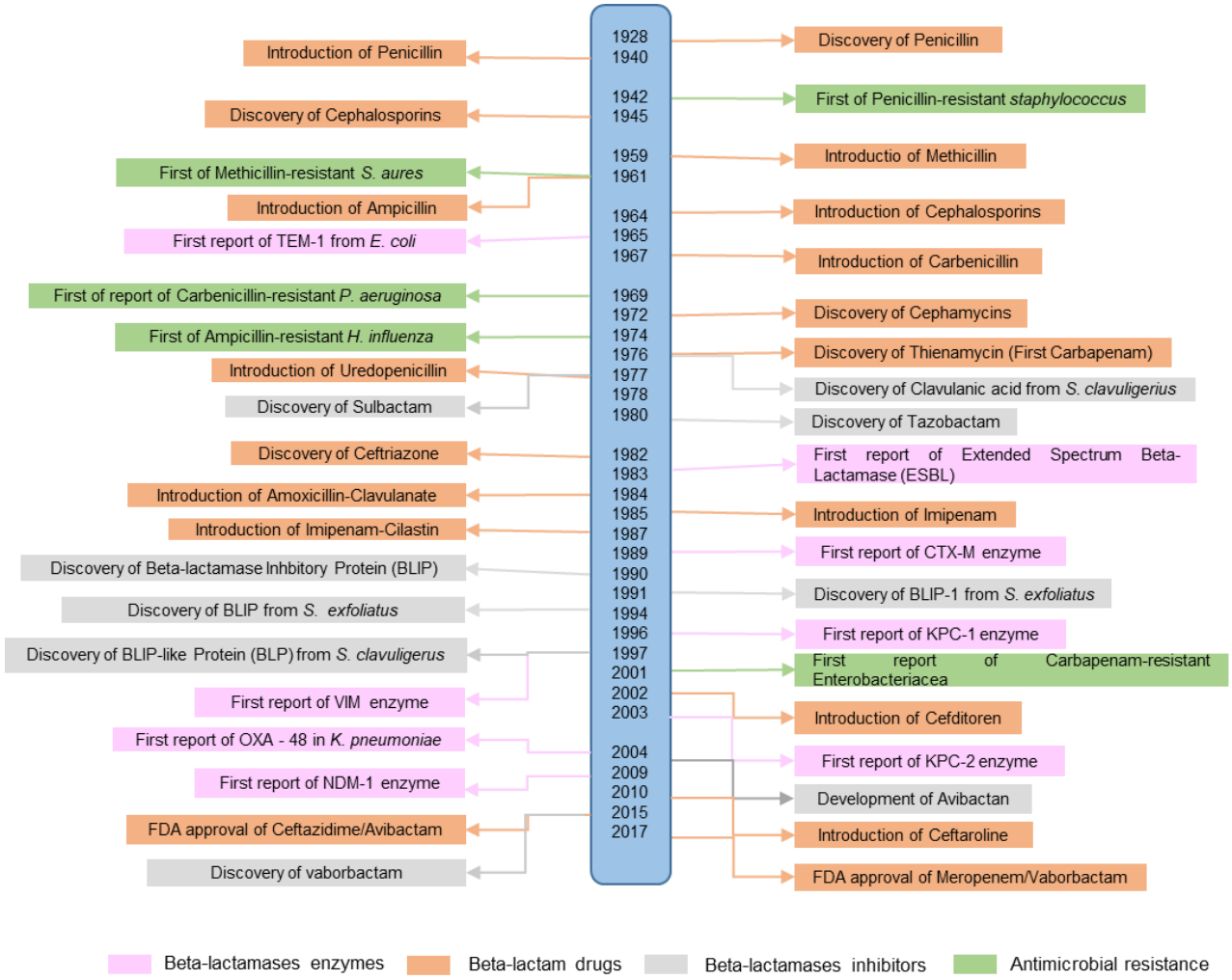


Figure 3: Discovery of β-lactams and BLIs and the development of resistance over the years (adapted from Eiamphungporn et al. 2018).

and the Bush classification, that is based on substrate and inhibition profile – functionality (Ambler 1980; Bush et al. 1995). In 1995, Bush et al. (1995) had proposed the junction of the Ambler and Bush classification and in 2010; this classification was update (Bush and Jacoby 2010). The functional classification is based on key β -lactam substrates and inhibitors which define three main β-lactamase classes and respective subclasses according to their hydrolytic and inhibitor profile (Bush and Jacoby 2010) The β-lactamases enzymes, accordingly with the Amber classification can be divided into four classes (classe A to classe D) (Table 1).

Since the discovery of penicillin (β-lactam) by Alexander Fleming in the late 1920s, several other β-lactams (cephalosporins, carbapenems and monobactams) have been developed for anti-infective therapy (Bush and Bradford 2016*).* β-lactams are among the most used antimicrobials in human and veterinary medicine, constituting 60% of worldwide antimicrobial usage, and are among the most effective antimicrobials for treatment of infection diseases (ECDC 2016; EMA 2017; Eiamphungporn et al. 2018) and are characterised by the presence of a β-lactam ring (Smet 2010).

In the 1970s, ampicillin and amoxicillin with improved activity against Enterobacterales Order were introduced (Bush and Bradford 2016). This group also includes anti-pseudomonal penicilins (carbenicillin, piperacillin, and ticarcillin), mecillinam and temocillin (Bush and Bradford 2016). Amoxicillin is excreted in urine predominantly in the active form (Weese et al. 2019), so aminopenicillin is the recommended for companion animal UTI treatment. The first cephalosporins were discovered in the 1950s (Asbel and Levison 2000; Fernandes et al. 2013). In veterinary medicine, the approved cephalosporins belong to the first to the fourth generations (Smet et al. 2010).

The Bush classification can be divided in three groups: group 1- cephalosporinases, group 2- serine β-lactamases and group 3- MBLs. In Gram-negative bacteria the emergence of ESBLs has been a major concern worldwide. ESBLs are characterized for the capacity to hydrolize third and fourth generation cephalosporins and monobactams but not cephamycins and carbapenems (Jacoby et al. 1988; Jarlier et al. 1988; Philippon et al. 1989; Bradford 2001).

The class A β-lactamases enzymes have broad substrate hydrolysis profiles, including penicillins, cephalosporins and some carbapenems. Class A β-lactamases enzymes included most of the ESBLs (functional group 2be group), including variants of TEM-1 (Temoniera), SHV-1 (sulfhydryl variable), following by CTX-M (cefotaximase) enzymes and KPC enzymes (*Klebsiella pneumonia carbapenemase*), with CTX-M being the most common (Philippon et al. 1989; Bradford 2001; Bonnet 2004; Jacoby 2009;

Table 1: β-lactamase classification (adapted from Bush and Jacoby 2010).

Functional group	Molecular class (subclass)	Distinctive substrate(s)	Inhibition		Defining characteristic(s)	Representative enzymes
			CA/TZB	EDTA		
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli Amp*C, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillin than cephalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime, and cefpirome	RTG-4

2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino--lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B (B3)					L1, CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Legend: CA, clavulanic acid; TZB, tazobactam.

Livermore 2009; Nordmann et al. 2011a; Zhao and Hu 2013). However, has the exception of OXA-type, enzymes (class D or oxacillinase). The first ESBLs were described in Europe in the 1980s, and since then ESBLs have been reported worldwide (Paterson and Bonomo 2005; Fernandes et al. 2013). CTX-M β-lactamases have high hydrolysis capacity against cefotaxime and are capable of higher cephalothin hydrolysis in comparison to penicillins, cefotaxime and ceftazidime (Tzouvelekis et al. 2000). CTX-M enzymes are present in various types of bacteria from all continents and have been recognized as the most frequent ESBL in Enterobacterales causing healthcare and community associated infections and is now endemic worldwide (Mathers et al. 2015; Bajaj et al. 2016). The CTX-M enzymes include at least six lineages (CTX-M-1-group, CTX-M-2-group, CTX-M-8-group, CTX-M-9-group, CTX-M-25-group, and KLUC-group) (Canton and Coque 2006; Mathers et al. 2015a; Bajaj et al. 2016). CTX-M phenotype has been reported in *E. coli* isolates from humans, companion animals, wildlife, food products and livestock, indicating the importance of the reservoirs harbouring and disseminating these enzymes (Hilty et al. 2012; Nicolas-Chanoine et al. 2014; Mathers et al. 2015a; Bajaj et al. 2016). There is host and geographic variation of the CTX-M enzymes distribution, some enzymes, such as CTX-M-15, are worldwide disseminated (Cantón and Coque 2006; Nicolas-Chanoine et al. 2008; Smet et al. 2010; Ewers et al. 2012; Belas et al. 2014; Bevan et al. 2017; Marques et al. 2018). CTX-M-15 enzyme appear to be more common in humans, but it is also frequently detected in companion animals (Coque, et al. 2008; Ewers et al. 2012). CTX-M-1 enzyme is the most frequently detected in bacteria from companion animals from Europe (Coque et al. 2008; Ewers et al. 2012; Belas et al. 2014; Marques et al. 2018). CTX-M-producing Enterobacterales, especially *E. coli* isolates are often co-resistant to other classes of antimicrobials, like fluoroquinolones, trimethoprim/sulfamethoxazole, and aminoglycosides leading to high rates of co-selection (Pitout and Laupland 2008; Bajaj et al. 2016). The CTX-M dissemination over the world has contributed to the rapid global increase in the rate of cephalosporin resistance among Enterobacterales with subsequent increased usage of the carbapenems for the medication of infections due to these MDR bacteria (Mathers et al. 2015a).

Additionally, to ESBLs, the Class C enzymes or AmpC type β-lactamases or Cephalosporinases (groups 1 and 1e) are commonly isolated from extended-spectrum cephalosporin resistant Gram-negative bacteria and are important causes of cephalosporin and cephamycin resistance. AmpC β-lactamases hydrolyze diverse β-lactam antibiotics, including cephamycins (cefoxitin), oxyimino cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) and monobactams (aztreonam). These enzymes are typically encoded on the chromosome and in plasmids (Bush and Jacoby

2010). In most Enterobacterales family, AmpC is inducible, unlike plasmid encoded AmpC (pAmpC) where the enzymes are mostly expressed constitutively (Jacoby 2009; Bush and Jacoby 2010). These include several enzymes families such as MOX, FOC, DHA, ACC, MIR, CMY, which are more hydrolytic than ESBL. These enzymes are usually resistant to clavulanic acid and active on cephamicyns (Bush and Jacoby 2010; Jacoby 2012). The most common AmpC cephalosporinases among commensal and pathogenic bacteria of humans and companion animals are CMY-2 and DHA-1 (Smet et al. 2010; Ewers et al. 2012; Belas et al. 2014; Marques et al. 2018). These enzymes have increase over the last ten years, however, are less frequently reported compared to ESBLs enzymes (Mairi et al. 2018). AmpC type β-lactamases are normally inactivated by boronic acid and avibactam (Drawz and Bonomo 2010).

During the XXth Century, this increase in Extended-Spectrum β-lactamases (ESBLs)-producing bacteria capable of hydrolysing almost all β-lactams antibiotics, namely the 3rd and 4th generation cephalosporins, except for the carbapenems. The consequence of this emerging phenomenon has been an increased consumption of carbapenems in human hospitals and with-it new resistance mechanisms.

Carbapenems (imipenem and meropenem), are CIA with resistance to most β-lactamases and have activity against Gram-positive and Gram-negative bacteria (Papp-Wallaceet al. 2011; Fernandes et al. 2013; Bush and Bradford 2016, Weese 2019). Since the introduction of imipenem in the 1980s, there have been various isolations of carbapenemase-producing bacteria (Nordmann et al. 2012). Carbapenemases are enzymes with the ability to hydrolyze carbapenems. They belong to three different molecular classes: The Ambler class A and class D; and Ambler class B or metallo-β-lactamases (MBL) (Queenan and Bush 2007). KPC β-lactamases can hydrolyze penicillins, cephalosporins, monobactams and carbapenems. Moreover, KPC-2 and KPC-3 are the most widespread variants of KPC β-lactamases. Class A enzymes can be inhibited by β-lactamase inhibitors (clavulanic acid, tazobactam and sulbactam), although KPC enzymes are not efficiently inhibited by these BLIs (Queenan et al. 2007; Nordmann and Poirel 2014). The first isolation of a KPC (KPC-3) in Portugal was in 2009 (Jorge da Silva and Duarte 2012). The class D oxacillin-hydrolyzing carbapenemases (OXA) are plasmid-mediated and have been identified mostly in multidrug-resistant isolates of *Acinetobacter baumannii* (Nordmann et al. 2012).

The Class B enzymes or metallo-β-lactamases (functional group 3a and 3b) have a broad substrate spectrum and can catalyze the hydrolysis of all β-lactams except for monobactams. Furthermore, they are only inactivated by metal chelators (ethylenediaminetetraacetic acid - EDTA) (Drawz 2010; Palzkill 2013). There are several types of Class B β-lactamases, including VIM (Verona integron-encoded metallo-β-

lactamase), IMP (imipenemase) and NDM-1 (New Delhi metallo-β-lactamase). The spread of the IMP- and VIM-type enzymes in Gram-negative pathogens was reported in the 1990s (Walsh et al. 2005).

Carbapanemen - resistant Enterobacterales are classified as an urgent clinical threat by the Centers for Disease Control and Prevention (CDC 2013) and as priority; pathogens for which new antimicrobials are urgently need by the World Health Organization (WHO 2019).

The use of carbapenems is not approved for use in food-producing animals in veterinary medicine because they play a vital role in the treatment of human clinical infections caused by multidrug resistant (MDR) gram negative bacteria, particularly those caused by ESBL-producing Enterobacterales or *Pseudomonas aeruginosa*. Although, their use can be exceptionally possible in veterinary medicine in companion animals in urinary or other bacterial infections by the agreement of a veterinary specialist/ pharmacologist (Weese et al. 2019) and under the compassion use still present in the European legislation (Directive 2001/82/EC; EMA 2018).

During last years, the prevalence of resistance to carbapenems has increase worldwide and has become a major public health problem. Moreover, ESBL and AmpC β - lactamases may also confer resistance to carbapenem when associated with other resistance mechanisms such porin deficiency or overexpression of efflux pumps (Bradford et al. 1997; Wozniak et al. 2012). In 1988, the first plasmidic carbapenemase, IMI-1, was reported in Japanese *Pseudomonas aeruginosa* isolate (Watanabe et al. 1991). However, the first carbapenemase producer in Enterobacterales (NmcA) was identified in 1993 in a clinical isolate of *Enterobacter cloacae* (Nordmann et al. 1993). This class A carbapenemase was chromosomally encoded but has rarely been reported. Subsequently, numerous carbapenemase-producing Enterobacterales have been reported in cases of nosocomial, community-acquired infections, livestock, companion animals, wildlife and environment (Köck et al. 2018). The number of reports of companion animals with infections (UTIs, and Skin and soft-tissue infections (SSTIs)) and colonization by carbapenemase-producing Gram-negative bacteria are increasing over the world. NDM-1 and OXA-48 producing *E. coli* have been detected in pathogenic bacteria causing clinical infections in dogs and cats in the USA and in dogs in Europe, respectively (Shaheen et al. 2013; Stolle et al. 2013) (Table 2). Carbapenem-resistant Enterobacterales in companion animals normally harbouring *bla*$_{\text{OXA-48}}$, *bla*$_{\text{NDM-1}}$ and *bla*$_{\text{NDM-5}}$ and the most common genes were found in *E. coli* and *Klebsiella pneumonia* (Shaheen et al. 2013; Stolle et al. 2013; Schmidel et al. 2014; Liu et al. 2016; Yousfi et al. 2016; Melo et al. 2017; Wang et al. 2017; Grönthal et al. 2018; Hong et al. 2018; Li et

Table 2: Occurrence carbapenem-resistant Gram-negative bacteria in companion animals.

Year(s) of sample collection	Country	Animal species	Isolation source	Carbapenem-resistant species/ carbapenemase detected	Reference
2018	Switzerland	Dogs and cats	Rectal swabs from hospitalized animals	*E. coli* (*bla*$_{OXA-181}$)	Nigg et al. 2019
2017	Thailand	Dog	UTI	*Acinetobacter baumannii* (*bla*$_{OXA-23}$)	Chanchaithong et al. 2018
2017	Korea	Dogs	Rectal swabs from hospitalized animals	*E. coli* (*bla*$_{NDM-5}$)	Hong et al. 2018
2016	Sebia	Dog	UTI	*Acinetobacter baumannii* (*bla*$_{OXA-72}$)	Misic et al. 2018
2016	Ohio	Dogs	UTI and infected bite wound by another dog	*E. cloacae* (*bla*$_{KPC-4}$)	Daniels et al. 2018
2016	Brazil	Dog	SSTIs	*Pseudomonas aeruginosa* (*bla*$_{VIM-2}$)	Fernandes et al. 2018a
2015-2017	Algeria	Pets	Fresh faecal samples from non-hospitalized animals	*Enterobacterales* (*bla*$_{OXA-48}$)	Mairi et al. 2019
2015-2016	UK	Dog	Faecal sample	*E. coli* (*bla*$_{NDM-5}$)	Reynolds et al. 2019
2015-2016	Algeria	Dogs and cats	Rectal swabs from healthy animals	*E. coli* (*bla*$_{OXA-48}$), *K. pneumonia* (*bla*$_{OXA-48}$), *E. cloacae* (*bla*$_{OXA-48}$)	Yousfi et al. 2018
2015	Finland	Dogs	SSTIs	*E. coli* (*bla*$_{NDM-5}$)	Grönthal et al. 2018
2015	France	Dogs and cats	Healthy dogs and cats	*E. coli* (*bla*$_{OXA-48}$)	Melo et al.2017

2015	Germany	Dogs and cats	Mouth and rectal swabs from non-hospitalized animals	*Acinetobacter baumannii* (*bla*$_{OXA-23}$)	Hérivaux et al., 2016
2015	China	Dogs	Rectal swabs from non-hospitalized animals	*E. coli* (*bla*$_{NDM-5}$)	Li et al. 2018
2014-2015	Italy	Dogs and cats	Rectal swabs from hospitalized animals	*Acinetobacter baumannii* (*bla*$_{OXA-23}$), *Acinetobacter radioresistens* (*bla*$_{NDM-1}$)	Gentilini et al. 2018
2014-2015	China	Dogs from farms	Rectal swabs	*E. coli* (*bla*$_{NDM-1}$, *bla*$_{NDM-5}$, *bla*$_{NDM-9}$)	Wang et al. 2017
2014-2015	Algeria	Dogs and cats	Rectal swab from a cat with RTI and from heathy animals	*E. coli* (*bla*$_{OXA-48}$, *bla*$_{NDM-5}$)	Yousfi et al. 2016
2014-2015	Germany	Dogs and cats	SSTIs and RTIs	*Acinetobacter pittii* (*bla*$_{OXA-58}$)	Klotz et al. 2017
2014-2015	Spain	Dog	Rectal swab from a healthy animal from shelter.	*K. pneumonia* (*bla*$_{VIM-1}$)	González-Torralba et al. 2016
NA	Australia	Cat	Rectal swab from a cat wih persistent haemorrhagic diarhoea - following treatment of an RTI	*Salmonella entérica* Typhimurium (*bla*$_{IMP-4}$)	Abraham et al. 2016
2013	China	Dog	NA	*E. coli* (*bla*$_{NDM-1}$)	Cui et al. 2018
2012-2016	Germany	Dogs and cats	UTI, SSTIs and others (e.g. blood, RTIs; CVCs)	*E. coli* (*bla*$_{OXA-48}$), *K. pneumonia* (*bla*$_{OXA-48}$)	Pulss et al. 2018
2012	Germany	Dogs	UTI, SSTI, RTI, faeces, CVC	*E. coli* (*bla*$_{OXA-48}$), *K. pneumonia* (*bla*$_{OXA-48}$)	Stolle et al. 2013
2009-2013	USA	Dogs and cats	Clinical isolates	*E. coli* (*bla*$_{OXA-48}$)	Liu et al. 2016
2009-2011	Germany	Dogs and cats	Clinical Isolates	*K. pneumonia* (*bla*$_{OXA-48}$)	Schmiedel et al., 2014
2009	Portugal	Cat	UTI	*Acinetobacter baumannii* (*bla*$_{OXA-23}$)	Pomba et al. 2014a

2008-2009	USA	Dogs and cats	UTI, RTI, SSTIs	*E. coli* (*bla*$_{NDM-1}$)	Shaheen et al. 2013
2000-2013	Germany	Dogs and cats	UTI, SSTIs and others (e.g. blood)	*Acinetobacter baumannii* (*bla*$_{OXA-23}$ and *bla*$_{OXA-66}$)	Ewers et al. 2017
2000	Germany	Cat	UTI	*Acinetobacter baumannii* (*bla*$_{OXA-23}$)	Ewers et al. 2016

Legend: NA- not available; CVC- central venous catheter; RTI- Respiratory tract infection; UTI-Urinary tract infection; SSTIs - Skin and soft-tissue infections.

al. 2018; Pulss et al. 2018; Wi et al. 2018; Yousfi et al. 2018; Mairi et al. 2019; Reynolds et al. 2019).

1.1.2 Fluoroquinolone resistance

After β-lactams, quinolones are among the most use antimicrobial in human medicine and in veterinary medicine, especially in companion animals (ECDC 2016; EMA 2017b). The first quinolone described was nalidixic acid in the 1960s for UTI treatment (Lesher et al. 1962). Nalidixic acid showed a narrow spectrum of activity, being active mainly against Enterobacteriales. It was the initial antimicrobial from which other quinolones generations were developed (Vila et al. 2016). Since then, several other quinolones were discovered including for veterinary use. Quinolones can be classified into first to fourth generation based on their spectrum of activity (Cotard et al. 1995; Weese et al. 2011). All quinolones are classified into different groups based on their chemical structure or their biological activities. All have a carboxylic substituent at position 3, which together with the carbonyl group at position 4, appears to be essential for the activity of the quinolones.

The second generation represented by ciprofloxacin and norfloxacin presented two main changes with respect to nalidixic acid, the first was a positively charged group at position 7 (piperazine group in ciprofloxacin) and a fluoride at position 6, therefore, since then the new quinolones have been called fluoroquinolones. This generation of quinolones was active against all aerobe gram-negative bacteria (Vila et al. 2016). The first fluoroquinolone approved for use in animals was enrofloxacin, which was approved for use in the United States in companion animals in 1988. Since the approval of enrofloxacin, other fluoroquinolones have been approved for use in companion and food animals (Giguère and Dowling 2013).

Quinolones inhibit the activity of the DNA gyrase and topoisomerase IV bacterial enzymes that are essential to regulate the topology of the bacterial chromosome and adequate DNA replication (Hooper and Jacoby 2015; Vila et al. 2016). The main function of the DNA gyrase is to catalyse negative supercoiling of the DNA, thereby playing an important role in DNA replication and transcription (Vila et al. 2016). Both enzymes are heterotetrameric proteins composed of two subunits consisting in two A subunits and two B subunits. The A subunit of the DNA gyrase is encoded by the *gyr*A gene and the B subunit by the *gyr*B gene. Topoisomerase IV also has a structure constituted by two A subunits and two B subunits. The A subunit is encoded in the *par*C gene and the B subunit in the *par*E gene (Hooper and Jacoby 2015; Vila et al. 2016).

Resistance to quinolones has steadily risen over the last decades. In Gram-negative bacteria, DNA gyrase is usually the main target (Vila et al. 2016).

Nowadays, two important mechanisms of resistance to quinolones can be found in *E. coli*: associated with mutations in the chromosome and related to plasmids (Table 3). However, in *E. coli* mutations in the *gyr*A and *par*C genes are one of the most important mechanisms of

resistance to quinolones. Mutations associated with resistance have been mapped in a region called the quinolone resistance determining region (QRDR) (Vila et al. 2016). Another mechanism of resistance to quinolones linked to mutations in the chromosome is the reduction in the intracellular accumulation of quinolones, which are related to a decrease in the permeability of the outer membrane or to an increased efflux of the antimicrobial out of the cell. The most important efflux pump is that encoded in the *acrAB* operon, in which the *acrB* gene encodes an inner membrane protein (AcrB), which is an efflux transporter across the inner membrane, and the *acrA* gene, which encodes a membrane fusion protein (AcrA). The third protein in this tripartite efflux pump is TolC which is an outer membrane protein. It has been shown that the overexpression of this efflux pump leads to a multidrug resistant phenotype in different bacteria, including *E. coli* (Vila et al. 2016). A chromosomal locus, called *mar* (multiple antibiotic resistance), encodes a transcriptional factor which increases *micF* expression, a regulatory antisense RNA, which causes a post-transcriptional decrease of OmpF RNA and reduces the amount of OmpF. MarA can regulate the expression of AcrAB-TolC which is increased when MarA is overexpressed (Vila et al. 2016). This relationship between the decreased expression of OmpF and the increased expression of ArcAB-TolC generates a multidrug-resistant phenotype in *E. coli* with increased resistance to quinolones, chloramphenicol and tetracyclines (Vila et al. 2016).

Table 3: Mechanisms of quinolone resistance in *Escherichia coli* (adapted from Vila et al. 2016).

1. Chromosomal-mediated	
Changes in protein targets	• Mutations in the *gry*A gene (amino acid codon Ser-83 and Asp-87)
	• Mutations in the parC gene (amino acid codon Ser-80 and Glu-84
Reduction in the accumulation of quinolone	• Decrease in permeability – Decreased expression of OmpF
	• Increase in active efflux systems: AcrAB, AcrEF, MdfA, YdhE
2. Plasmid-mediated	
DNA gyrase and topoisomerase IV protection from quinolone inhibition - Qnr	
Aminoglycoside-acetyltransferase – AAC (6') -Ib-cr	
Efflux pumps: QepA and OqxAB	

Until now, three plasmid-mediated mechanisms of resistance to quinolones have been described (Table 3):

1) Plasmid-encoded Qnr proteins (small pentapeptide-repeat proteins) bind to the topoisomerase targets, protecting them from quinolones. The qnr gene family, which encode a peptide able to protect the DNA gyrase or DNA-topoisomerase IV complexes to be bound by the quinolones (Tran and Jacoby 2002; Vila et al. 2016). Several plasmid-encoded qnr alleles (QnrA, QnrB, QnrC, QnrD, QnrS and QnrVC) and multiple variants have been described in Gram-negative bacteria (Vila et al. 2016).

2) The expression of an aminoglycoside-modifying enzyme (AAC (6′) Ib-cr) which has the capacity to acetylate an amino group located in the piperazine ring of the quinolone structure, such as ciprofloxacin (Robicsek et al. 2006; Vila et al. 2016).

This resistance mechanism is worldwide disseminated in two gram negative bacteria, *E. coli* and *K. pneumoniae* and it seems to be the most common plasmid mediated quinolone resistance (PMQR) determinant (Park et al. 2006; Robicsek et al. 2006; Pitout et al. 2008; Vieira et al. 2020). These PMQRs determinants may be encoded in plasmids harbouring ESBL and other resistance genes, so, the use of quinolones may contribute to the co-selection of ESBLs and other resistance determinants and vice versa (Jacoby et al. 2014). PMQRs are found over the world in Enterobacterales isolated from humans and companion animals (Jacoby et al. 2014).

3) Efflux pumps (*Oqx*AB and *Qep*A). These pumps affect small increases in the Minimal Inhibitory concentrations (MICs) of quinolones but are enough to occur the selection of mutants with higher levels of resistance, especially in the *gyrA* gene (Vila et al. 2016). These increase the resistance to fluoroquinolones such as: norfloxacin, ciprofloxacin, and enrofloxacin, and also to other antimicrobial classes (Vila et al 2016).

Fluoroquinolones are highest priority critical important antimicrobials (HP-CIA) for humans (WHO 2019) and have been shown to increase the risk for selection of resistant bacteria. The use of fluoroquinolones in companion animals should be reserved to resistant infections but can be first line choice for pyelonephritis and for infections that involve the prostate (Weese et al. 2019).

1.1.3 Aminoglycosides resistance

Aminoglycosides were identified in the 1940s and are natural or semisynthetic antimicrobials derived from *actinomycetes*. The first aminoglycoside streptomycin was discovered from *Streptomyces griseus* (Doi et al. 2016; Krause et al. 2016). They were among the first antibiotics to be introduced for routine clinical use and several examples have been approved for use in humans, such as neomycin (1949), kanamycin (1957), gentamicin (1963), netilmicin (1967), tobramycin (1967) and amikacin (1972) (Krause et al. 2016). They found

widespread use as first-line agents in the early days of antimicrobial therapy, but were replaced in the 1980s with cephalosporins, carbapenems, and fluoroquinolones (Doi et al. 2016; Krause et al. 2016). However, increasing resistance to these antimicrobials, combined with the knowledge of the basis of aminoglycoside resistance, has led to renewed interest in the legacy and development of aminoglycosides (Krause et al. 2016). Moreover, they are particularly clinically important, especially gentamicin, tobramycin and amikacin, for the treatment of MDR-Enterobacterales infection and in critical care of humans (Krause et al. 2016). Furthermore, aminoglycosides are CIAs of high priority to humans (WHO 2019). Regarding companion animal, amikacin is not recommended for routine use, but can be used for the treatment of MDR bacteria yet must be avoid in animals with reduced kidney function, because this antimicrobial is a potentially nephrotoxic (Weese et al. 2019).

Aminoglycosides are potent, broad-spectrum antimicrobials that act through inhibition of protein synthesis by binding, with high affinity, to the aminoacyl-tRNA recognition site (A-site) on the 16S ribosomal RNA (16S rRNA) of the 30S ribosomal subunit ribosome, leading to inhibition of polypeptide synthesis and subsequent cell death (Doi et al. 2016; Krause et al. 2016). Although aminoglycoside class members, have a different specificity for different regions on the A-site. The mechanism of binding and the subsequent downstream effects varies by chemical structure. Therefore, some aminoglycosides promote mistranslation by inducing codon misreading on delivery of the aminoacyl transfer RNA, which can lead to inhibition of protein synthesis and aminoglycosides antimicrobials access to the cytoplasm, which leads to cell membrane damage and death (Krause et al. 2016). Other aminoglycosides can also affect protein synthesis by directly inhibiting initiation or blocking elongation (Krause et al. 2016).

A typical aminoglycoside possesses an amino-containing or non-amino-containing sugar linked to six-membered rings with amino group substituents, are characterized by a core structure of amino sugars connected via glycosidic linkages to a dibasic aminocyclitol, which is most commonly 2-deoxystreptamine (Doi et al. 2016; Krause et al. 2016). Aminoglycosides are broadly classified into four subclasses based on the identity of the aminocyclitol porcion: no deoxystreptamine ring (streptomycin); a mono-substituted deoxystreptamine ring (apramycin); 4,5-di-substituted deoxystreptamine ring (neomycin, ribostamycin) and 4,6-di-substituted deoxystreptamine ring (gentamicin, amikacin and tobramycin) (Krause et al. 2016). The variety of amino and hydroxyl substitutions have a direct influence on the mechanisms of action and susceptibility to various aminoglycoside-modifying enzymes (AMEs) associated with each of the aminoglycosides (Krause et al. 2016).

Aminoglycosides resistance can occur by the following mechanisms including enzymatic modification and inactivation of the aminoglycosides mediated by aminoglycoside acetyltransferases, nucleotidyltransferases, and phosphotransferases; target site modification via an enzyme (16S rRNA methylases) or chromosomal mutation; increased efflux; decreased

permeability; and by modifications of the 30S ribosomal subunit that interferes with binding of the aminoglycosides (Doi et al. 2016; Krause et al. 2016). However, the most common aminoglycoside resistance mechanism is the production of Aminoglycoside-modifying enzymes (AMEs) (Garneau-Tsodikova and Labby 2016).

A diversity of AMEs were identified and are categorized into three groups based on the capacity to acetylate, phosphorylate and adenylate amino or hydroxyl groups. These three groups include aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-nucleotidyltransferases (ANTs), and aminoglycoside O-phosphotransferases (APHs) (Krause et al. 2016). AMEs group includes several enzymes with different spectrums of activity. Among the high number of AMEs described until now, the AAC (6')-Ib, is the most prevalent and clinically relevant AME in Enterobacteriales family (Ramirez et al. 2013). Several AAC (6')-Ib enzyme variations have been described including the widely disseminated ciprofloxacin modifying variant AAC (6')-Ib-cr (Krause et al. 2016).

AMEs are often found in mobile elements such as plasmids and may containing other additional resistance elements, including β-lactamases, such as ESBLs and carbapenemases (Krause et al. 2016).

The ribosomal binding site may also be modified enzymatically by 16S ribosomal RNA methyltransferases (RMTs) (Krause et al. 2016). The 16S rRNA methylases modify specific rRNA nucleotide residues in a way that blocks aminoglycosides to binding to their target. RMTs can be characterized into two classes, according to the nucleotide position where methylation occur (Garneau-Tsodikova and Labby, 2016; Krause et al. 2016). These enzymes can be plasmid mediated in Enterobacterales and its dissemination is a concern (Krause et al. 2016). These enzymes have been described worldwide in Enterobacterales isolates from human, livestock sources and companion animals, with ArmA and RtmB as the most frequent (Deng et al. 2011; Wachino and Arakawa 2012; Hidalgo et al. 2013; Krause et al. 2016, Xia et al. 2017). Moreover, the association between RMTs and ESBLs and carbapenemases has also been described in Enterobacterales isolates from human (Berçot et al. 2011; Livermore et al. 2011; Poirel et al. 2014).

1.1.4 Trimethoprim/sulfamethoxazole resistance

In the early 1970s, trimethoprim/sulfamethoxazole demonstrated a wide spectrum of activity against aerobic bacteria (Masters et al. 2003). Trimethoprim-sulfamethoxazole is active against many Enterobacterales and is considered useful for UTI treatment in humans since 1968 (Eliopoulos and Huovinen 2001). However, increasing rates of resistance among clinically important pathogens have been reported worldwide during the past few decades (Masters et al. 2003). The trimethoprim/sulfamethoxazole combination is considered highly important antimicrobial (HIA) (WHO 2019), however is an appropriate initial or empirical option for UTI

treatment in companion animals and can be considered a treatment choice for prostate infections (Weese et al. 2019). Trimethoprim/sulfamethoxazole in combination is thought to have a synergistic effect and both affect bacterial folic acid synthesis, the physiologically active form of folic acid and a necessary cofactor in the synthesis of thymidine, purines, the fundamental bases of bacterial DNA and RNA (Eliopoulos and Huovinen, 2001; Masters et al. 2003; Wüthrich et al. 2019). These antimicrobials act at different steps of the bacteria tetrahydrofolic acid biosynthesis, which is essential for bacteria amino acid and nucleotide synthesis (Eliopoulos and Huovinen, 2001). Sulphonamide is a structural analogue of para-aminobenzoic acid (PABA) and inhibit dihydropteroate synthetase (DHPS), which catalyses the formation of dihydrofolate from para-aminobenzoic acid. Trimethoprim is a structural analogue of the pteridine portion of dihydrofolic acid that competitively inhibits dihydrofolate reductase (DHFR), which catalyses the formation of tetrahydrofolate from dihydrofolate. This sequential blockade of two enzymes in one pathway results in an effective bactericidal action (Eliopoulos and Huovinen 2001; Masters et al. 2003; Wüthrich et al. 2019). Bacteria may become resistant to trimethoprim/sulfamethoxazole by different mechanisms: 1) development of permeability barriers and/or efflux pumps, 2) a naturally insensitive intrinsic (DHFR), 3) spontaneous chromosomal mutations in the intrinsic (DHPS) (*fol*P) and DHFR (f*ol*A) genes involved in the folic acid pathways, 4) increased production of the sensitive target enzyme by upregulation of gene expression or gene duplication and 5) the acquisition of alternative DHPS (*sul*) and DHFR (*dfr*) genes with integrons, plasmids, and transposons (Eliopoulos and Huovinen 2001; Masters et al. 2003; Wüthrich et al. 2019). Resistance to sulphonamides in *Enterobacterales*, such *E. coli* can result from chromosomal mutations in the DHPS gene (*fol*P) (Swedberg et al. 1993; Vedantam et al. 1998) or caused by plasmid borne mechanisms, the acquisition of an alternative DHPS gene (*sul*) (Rådström and Swedberg 1988; Sundström et al. 1988; Sköld*,* 2000), with low sulphonamide affinity encoded in mobile elements (Swedberg and Sköld. 1980). The first transferable sulphonamide resistance genes in Enterobacterales family, *sul*1 and *sul*2, were described in the 1980s (Rådström and Swedberg 1988; Sundström et al. 1988) and only in 2003 *sul*3 gene has been discovered (Perreten and Boerlin 2003). The *sul*1 gene is usually found in class 1 integron, while *sul*2 is usually located on small plasmids belonging to the IncQ family or another type represented by pBP1 or in large transmissible multiresistance plasmids (van Treeck et al. 1981; Huovinen et al. 1995; Sköld 2000; Enne et al. 2001). The *sul*3 gene linked to class 1 integrons lacking their 3´ CS region (Bean et al. 2009). Integrons are composed of two conserved DNA regions, located at their ends, which are known as 5´ CS and 3´ CS (5´ and 3´ conserved segments). The acquisition and dissemination of these genes located within the integron structure results in an increase in antimicrobial resistance. Three classes of integron structure have been described (Cambray et al. 2010; Deng et al. 2015). However, the class 1 integrons are the most worrying in clinical isolates. The 5´-CS of class 1 integrons includes an *intI1* gene, which encodes a site-

specific tyrosine recombinase, which performs integration and excision of genetic elements, known as gene cassettes, at the *att1* recombination site. Usually, *Int*1 gene was associated with *sul* genes. The 3´CS region contains several open reading frames (ORFs) (Carattoli 2001; Partridge et al. 2009). These include *qacEΔ1*, which confers resistance to quaternary ammonium compounds, often associated with antiseptics, along with a *sul*1 gene expressing resistance to sulfonamides (Paulsen et al. 1993; Partridge et al. 2009).

The integrons are usually located on mobile genetic elements like plasmids and transposons, providing the ability of dissemination of *sul* genes between commensal bacteria into more virulent bacteria, and species (Carattoli 2001, Mazel 2006).

Both *sul*1 and *sul*2 are highly prevalent among uropathogenic *E. coli* isolates from humans, whilst the third type *sul*3 is less common (Blahna et al. 2006). In 2017, the fourth mobile sulphonamide resistance gene (*sul*4) was discovered by next generation sequencing (NGS) (Razavi et al. 2017). However, until now little is known about *sul*4.

Resistance to trimethoprim is mainly mediated by a dihydrofolate reductase enzyme encoded by the *dfr* genes, which are usually associated with class 1 and class 2 integrons or plasmids, which has led to the rapid emergence of trimethoprim resistance among bacterial populations (Yu et al. 2004; Ho et al. 2009). Different types of trimethoprim-resistant *dfr* genes and *dfr* gene cassettes have been detected in class 1 integrons (Yu et al. 2004; Ho et al. 2009; Wüthrich et al. 2019).

This association of *sul* and *dfr* genes with mobile genetic elements such as plasmids and integrons, is highly relevant for the increase in the emergence, evolution, and dissemination of sulphonamide resistance in environments (Yu et al. 2004). *E. coli* isolates from clinical specimens are mostly MDR and a high frequency of *E. coli* isolates from the urinary tract are resistant to trimethoprim (Huovinen 1995; Lee et al. 2001; Yu et al. 2004).

1.1.5 Tetracycline resistance

Tetracyclines were discovered in the 1940s and exhibited activity against a wide range of microorganisms including gram-positive and gram-negative bacteria. They are inexpensive antimicrobials, which have been used extensively in veterinary medicine, especially in food animals, but also in the prophylaxis and therapy of human and animal infections (Chopra and Roberts 2001, Grossman 2016).

Among tetracyclines, doxycycline is categorized by WHO (WHO 2019) as a HIA in human medicine, however, can be used for the treatment of UTIs in companion animals, but not as a first line option (Weese et al. 2019). Tetracyclines are bacteriostatic broad-spectrum antimicrobials that bind to bacterial ribosomes and interact with a highly conserved 16S ribosomal RNA (rRNA) target in the 30S ribosomal subunit (A-site) and inhibit bacterial protein synthesis

(Chopra and Roberts 2001; Grossman 2016). With the widespread use of tetracyclines after their discovery in the 1940s, resistance started to increase (Chopra and Roberts 2001). Tetracyclines resistance is normally attributed to three general class-specific mechanisms: efflux systems, ribosomal target protection, and enzymatic inactivation of tetracycline drugs (Grossman 2016). The acquisition of mobile genetic elements carrying tetracycline-specific resistance genes, mutations within the ribosomal binding site, and/or chromosomal mutations leading to increased expression of intrinsic resistance mechanisms (Grossman 2016).

Active efflux of tetracycline from the bacterial cell is mediated by membrane-bound efflux proteins encoded by several *tet* gene groups. Efflux proteins of group 1, including tet(A) and tet(B), are widely distributed in Gram-negative bacteria (Chopra and Roberts 2001; Roberts 2005). The gram-negative efflux genes are normally associated with plasmids and most of them are conjugative and often carry other antimicrobial resistance genes (Chopra and Roberts 2001). In Gram-negative bacteria such as *E. coli*, tetracycline passively diffuses through the outer membrane porins OmpF and OmpC, most likely as a Mg^{2+} chelate, and this is consistent with the finding that outer membrane porin mutants show resistance increase to tetracyclines (Chopra and Roberts 2001; Grossman 2016). Futhermore, Tetracyclines can also be inhibited by the production of cytoplasmic proteins that protect the ribosomes, most ribosomal protection proteins are encoded by *tet* genes, with the exception of tet(B) (Chopra and Roberts 2001).

1.1.6 Nitrofurantoin resistance

Nitrofurantoin was discovery in the 1940s and has been approved and available since 1953 for the treatment of lower urinary tract infection (UTI) (Huttner et al. 2015; Gardiner et al. 2019). However, this use decreases in the 1970s with the appearance of the β-lactam and trimethoprim/sulfamethoxazole antimicrobials (Huttner et al. 2015). Yet, in the last years with the increase of trimethoprim/sulfamethoxazole, fluoroquinolones and β-lactams resistance and also the emergence in the late 2000s of ESBL-producing and carbapenem-resistant bacteria, several guidelines were revised to put back on the market again the nitrofurantoin as first-line therapy for uncomplicated lower UTI (Huttner et al. 2015).Nitrofurantoin is a synthetic antimicrobial created from furan and an added nitro group and a side change containing hydantoin and it is effective against most gram-positive and gram-negative bacteria (Huttner et al. 2015; Gardiner et al. 2019). Some guidelines (Bekford-Ball 2006; AFSSAPS 2008) have declared nitrofurantoin as the first-line therapy for treatment of uncomplicated lower urinary tract infections in human medicine (Huttner et al. 2015; Gardiner et al. 2019; Squadrito and Portal 2019). Recently, accordingly with the World Health Organization it was been are categorized as an important antimicrobial (IA) for human medicine (WHO, 2019). Nitrofurantoin uses several mechanisms to achieve an antimicrobial effect. Nitrofurantoin is taken up by bacterial intracellular nitroreductases to produce the active form of the drug via reduction of the nitro group (Squadrito and Portal 2019).

Nitrofurantoin requires reduction by bacterial enzymes producing 'highly reactive electrophilic' metabolites, which inhibit protein synthesis by interfering with bacterial ribosomal proteins (Gardiner et al. 2019). Intermediate metabolites that result from this reduction then bind to bacterial ribosomes and inhibit bacterial enzymes involved in the synthesis of DNA, RNA, cell wall protein synthesis, and other metabolic enzymes. However, Nitrofurantoin mechanism action remains poorly understood (Gardiner et al. 2019; Squadrito and Portal 2019).

Nitrofurantoin resistance is uncommon and mostly of the MDR bacteria are susceptibility (Sanchez et al. 2016). However, nitrofurantoin resistance seems to be associated with chromosomal mutations, the *Oqx*AB plasmid mediated efflux pump in *E. coli* (Ho et al. 2016). Nitrofurantoin in veterinary medicine only can be used as an option (off-label use), is recommended by Weese et al. (2019) guidelines for the treatment of sporadic bacterial cystitis (uncomplicated UTIs), especially when MDR bacteria are involved (Weese et al. 2019).

1.1.7 Fosfomycin resistance

Fosfomycin was introduced in Europe throughout the 1970s and is a phosphonic acid derivative with broad-spectrum of antimicrobial activity, which inhibits cell wall and early murein/peptidoglycan synthesis (Gardiner et al. 2019). Fosfomycin is a safe and effective antimicrobial for urinary tract infections in humans with good tolerance, good tissue penetration and may provide an alternative option for the treatment of uncomplicated cystitis caused by ESBL-producing and carbapenemase-producing Enterobactereals, but its use should be limited to delay the development of resistance (Gardiner et al. 2019; Múñez Rubio et al. 2019). Fosfomycin even has activity against some types with no other available effective antibiotics and can act synergistically with other antibiotics (Rubio et al. 2019). Fosfomycin acts by inhibiting UDP-N-acetylglucosamine enolpyruvyl transferase (*mur*A), which prevents the formation of N-acetylmuramic acid, an essential component of peptidoglycan (Doesschate et al. 2019). Fosfomycin resistance mechanisms have been described involving decreased antimicrobial uptake, modification of the target site and antimicrobial inactivation (Karageorgopoulos et al. 2012). Several of these resistance mechanisms result from chromosomal mutations, some inactivation enzymes may be plasmid mediated, such as *fosA, fosA3, fosC2* and *fosK* (Yao 2016). Some studies indicated that mostly the plasmid encoded f*osA*3 gene mediates fosfomycin resistance. Moreover, this gene has been described in Enterobacterales isolates from humans, companion animals and livestock (Yao et al. 2016; Gardiner et al. 2019). *Fos*A is a glutathione transferase that inactivates fosfomycin through catalysing the addition of glutathione. *fos*A genes are often present in the chromosome of *Klebsiella pneumoniae*, but not in the chromosome of *E. coli* (Doesschate et al. 2019). However, resistance to fosfomycin is still low in *E. coli*; the acquisition of *fos*A may reduce future activity of fosfomycin to treat infections caused by *E. coli* (Doesschate et al. 2019). However, horizontal spread of *fos*A has been demonstrated, resulting

in development of fosfomycin resistance, the long-term use of oral fosfomycin can promote horizontal gene transfer from commensal gut flora to potential pathogenic bacteria, such as *E. coli* (Doesschate et al. 2019).

Fosfomycin is a CIA for humans (WHO 2019) and the use in human medicine is increasing due to the emergence of MDR bacteria (Doesschate et al. 2019). Yet, in veterinary medicine only can be used as an option (off-label use) and is recommended by Weese et al. (2019) guidelines for the treatment of multidrug resistant infections only in dogs with pyelonephritis and prostatitis, especially when MDR bacteria are involved.

1.1.8 Chloramphenicol and florfenicol resistance

Chloramphenicol and florfenicol are bacteriostatic, broad-spectrum antimicrobials that inhibit protein synthesis by binding to the 50S ribosomal subunit of the bacterial ribosome, inhibiting peptidyl transferase and preventing the amino acid transfer to growing peptide chains and subsequently inhibiting protein formation (Schwartz et al. 2004). Both antimicrobials have great activity against Gram-positive and Gram-negative, aerobic and anaerobic bacteria (Schwarz et al. 2004). Chloramphenicol is a broad-spectrum antimicrobial that has been used for several years in human and veterinary medicine (Schwarz et al. 2004). Florfenicol, on the other hand, is licensed exclusively for veterinary use in farm animals (Schwarz et al. 2004). Chloramphenicol causes a number of adverse effects, including dose-unrelated irreversible aplastic anaemia in humans and dose-related reversible bone-marrow suppression, and for this reason it is reserved for MDR life-threatening infections (Schwarz et al., 2004). (Schwarz et al. 2004). In small animals, chloramphenicol is used for ocular infections, otitis (Guiguère et al. 2013) and is reserved for the treatment of UTI caused by MDR bacteria in companion animals (Weese et al. 2019). Regarding florfenicol, this antimicrobial does not cause adverse side effects like chloramphenicol and so it is licensed for the control of bacterial respiratory tract infections in cattle and pigs (Schwarz et al. 2004).

The most frequently mechanism of bacterial resistance to chloramphenicol is enzymatic inactivation by acetylation of the drug by different chloramphenicol acetyltransferases (CATs). However, CAT enzymes do not inactivate Florfenicol (Schwartz et al. 2004; van Duijkeren et al. 2018). Acetylation of the hydroxyl groups on chloramphenicol prevents drug binding to the ribosomal subunit. Resistance may also be caused by other mechanisms such as efflux systems, inactivation by phosphotransferases and mutations of the target site and permeability barriers (Schwartz et al. 2004; van Duijkeren et al. 2018). The CAT genes are commonly detected on plasmids in *Enterobacterales* and most of these plasmids carry one or more additional resistance genes (Schwartz et al. 2004). Yet, the efflux of chloramphenicol and florfenicol from bacteria can be mediated by specific exporters, such as CmlA and floR or by multidrug exporters (Schwartz et al. 2004; van Duijkeren et al. 2018). The gene *cml*A is a Tn*1696*-associated cassette-borne,

which is inducible expressed via translational attenuation (van Duijkeren et al. 2018). Genes related to *cml*A and *flo*R are mainly found in Enterobacterales, such as *E. coli* and *klebsiella* spp. (van Duijkeren et al. 2018).

Multidrug exporters systems that export chloramphenicol have been described in *Enterobacterals*, including AcrAB/TolC efflux system in *E. coli* (Duijkeren et al. 2018). Moreover, the permeability barriers have also been described to confer resistance to chloramphenicol. The *mar* locus is found in *Enterobacterals* and can contribute to resistance by: 1) it can activate the AcrAB/TolC, leading to increase chloramphenicol efflux system; 2) MarA can activate the *micF* gene, which results in a decrease of chloramphenicol influx (van Duijkeren et al. 2018).

1.2 Antimicrobial resistance surveillance in companion animals in Portugal and Europe

There is growing concern globally about the misuse of antimicrobials. International meetings on this topic have supported a "One Health" approach that is defined as "a collaborative, multi-sectoral and trans-disciplinary approach, working locally, regionally, nationally and globally, to achieve optimum health and well-being of animals, people, plants and the environment shared by all, recognizing their inextricable interrelationships (One Health Commission 2018). This approach places the responsibility for the protection of antimicrobials in both human and veterinary medicine, by preserving the effectiveness of existing antimicrobials through their rational use (McEwen and Collignon 2017).

In recent years there has been growing concern about the role of companion animals as reservoirs of antimicrobial resistance (Guardabassi et al. 2004; Damborg et al. 2016; Pomba et al. 2017). The increase of antimicrobial resistance in companion animals represents a challenge in veterinary medicine as it limits therapeutic options, but also a potential vehicle for the spread of antimicrobial resistance genes (Bogaerts et al. 2015). We have been confronted with the emergence of methicillin-resistant *staphylococcus* spp. and Gram-negative MDR bacteria and several studies have shown the possibility of transmitting some strains of bacteria from man to dog and cat and vice versa (Guardabassi et al. 2004; Johnson et al. 2000; Johnson et al. 2008). Regarding antimicrobial resistance, some studies have been carried out on the topic and antimicrobial resistance may vary according to the geographic location (Marques et al. 2016; Moyaerts, 2017), moreover, their comparison is not simple, due to differences in study design, such as variations in host species, inclusion criteria and/or time period (Marques et al. 2016).

The European Antimicrobial Resistance Surveillance Network (EARS-Net) actively gathers and reports annual data on antimicrobial resistance in human invasive bacteria from several European countries (ECDC 2017). These EARS-Net reports show remarkable geographical differences in antimicrobial resistance frequencies among European countries as well as increasing trends in resistance to CIAs (ECDC 2017). Such important surveillance

programs are lacking in small animal veterinary medicine. There have been only few national antimicrobial resistance surveillance networks in place for companion animals in Germany, Sweden and France (Swedres-Svarm 2016; Moyaert et al. 2017). In 2008, the European Animal Health Study Centre started an initiative (Compath) gathering bacterial isolates from companion animals in Europe and just recently published data regarding UTI isolates from 2008-2010 (Moyaert et al. 2017). Moyaert et al. (2017) reported overall high susceptibility to all tested antimicrobials (e.g. >90% for most antimicrobials in *E. coli*). However, since the antimicrobial resistance frequencies were presented for all countries as a group and temporal trends were not analysed (Moyaert et al. 2017), it was not possible to perceive any geographical differences. Overall high antimicrobial susceptibility frequencies were also detected in previously published data from Sweden (2009, 2014) (Windahl et al. 2014; Swedres-Svarm 2016), Norway (2003-2009) (Lund et al. 2014) and Switzerland (*E. coli*, 1999-2001) (Lanz et al. 2003).

Under the umbrella of the European Society of Veterinary Nephrology and Urology, a multicenter retrospective study was launched in November 2013 with the goal of getting antimicrobial resistance data on bacteria isolated from companion animal with UTI across Europe (Marques et al. 2016). In this study participated the following countries: Germany, Austria, Belgium, Denmark, Spain, France, Greece, Holland, Italy, Portugal, United Kingdom, Serbia, Sweden and Switzerland, the most common etiology of UTI in companion animals (dogs and cats) was *E. coli* (Marques et al. 2016). One of the conclusions to be highlighted was that, for all bacteria, the countries of the South (Italy, Greece, Portugal and Spain) had higher levels of resistance for the studied antimicrobials (amoxicillin - clavulanic acid, 3GC, fluoroquinolones, gentamicin and trimethoprim - sulfamethoxazole) when compared to the countries of the North (Denmark, Sweden). Furthermore, MDR - *E. coli* was more frequent, also in the countries of the South (Marques et al. 2016) (Figure 4).

E. coli is the most frequent isolated bacteria causing UTI in companion animals (dogs and cats) and some studies show frequencies greater than 30 % (Marques et al. 2016). However, other commonly isolated bacteria genera include Staphylococcus spp., *Enterococcus* spp., *Proteus* spp. and *Klebsiella* spp. (Marques et al. 2016). Furthermore, to better understand the antimicrobial resistance temporal trends in uropathogenic bacteria from companion animals in Portugal (Lisbon), was performed a study by Marques et al. (2018) and notably, a significant increase in Enterobacterales antimicrobial resistance to the main antimicrobials used for UTI treatment in small animal veterinary medicine was observed in companion animals from Portugal (Lisbon), also for *E. coli* there was a significant increase in antimicrobial resistance to all antimicrobials, except trimethoprim/sulfamethoxazole (Figure 5) (Marques et al. 2018).

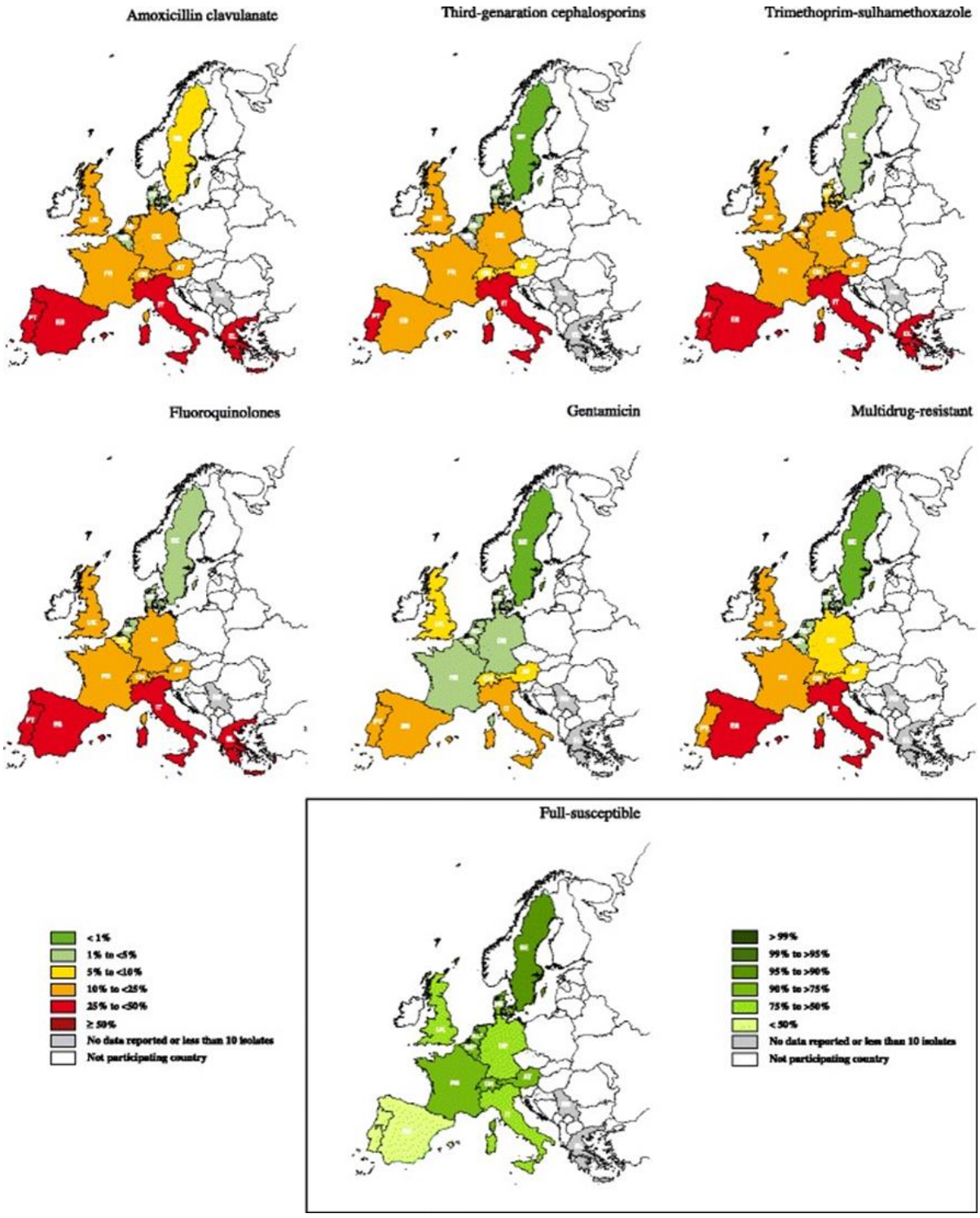


Figure 4: *Escherichia coli* antimicrobial resistance by antimicrobial in Europe in the years 2012–2013 (from Marques et al. 2016 with permission from the author).

Legend: Percentage (%) of *E. coli* antimicrobial resistance by antimicrobial and country in the years 2012–2013. Countries: AT- Austria; BE- Belgium; DK- Denmark; FR- France; DE- Germany; EL- Greece; IT- Italy; NL- the Netherlands; PT- Portugal; RS- Serbia; ES- Spain; SE- Sweden; CH- Switzerland; UK- United Kingdom.

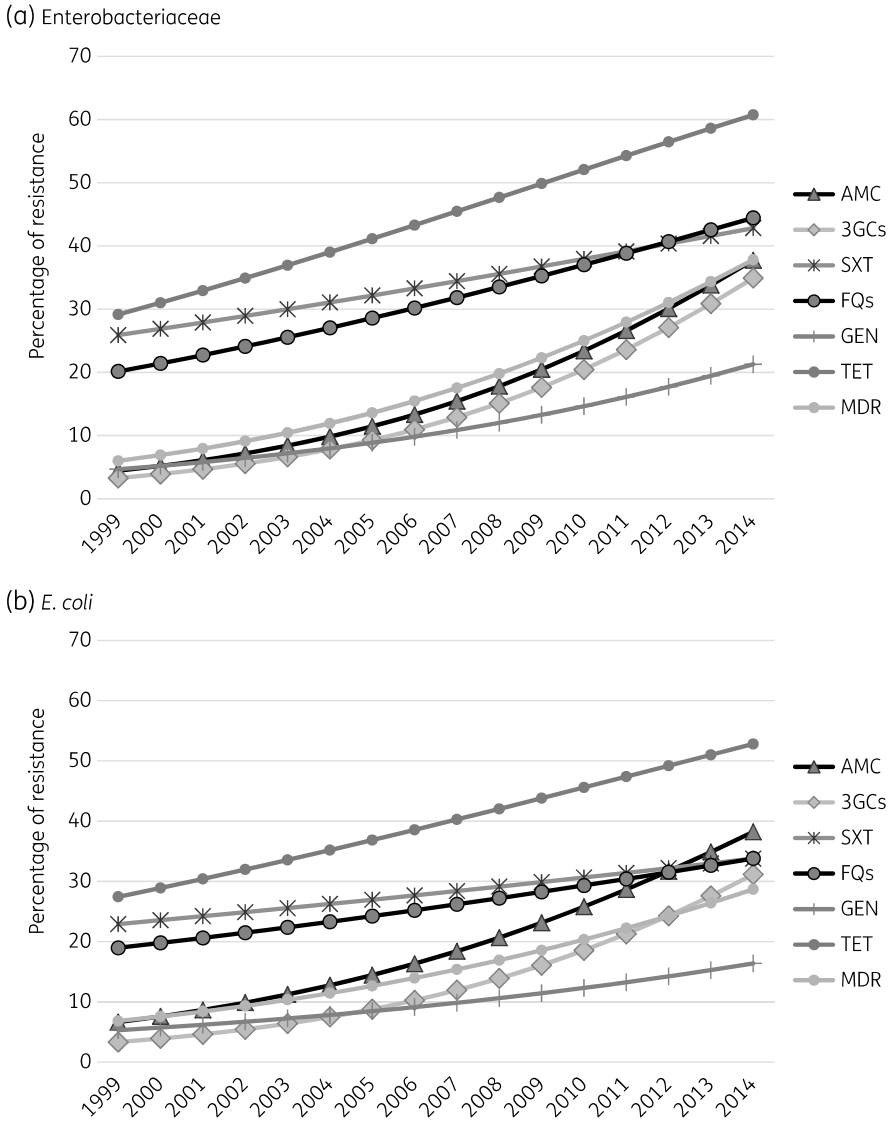


Figure 5: Enterobacteriacea and *Escherichia coli* resistance trends over the 16 years of the study (1999-2014) (adapted from Marques et al. 2018, with permission from the author).

Legend: (a) Enterobacteriaceae. There was a significant increase in antimicrobial resistance to all antimicrobials. (b) *E. coli*. There was a significant increase in antimicrobial resistance to all antimicrobials, except trimethoprim/sulfamethoxazole. AMC, amoxicillin/clavulanate; SXT, trimethoprim/sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline (Marques et al. 2018).

1.3 Risk of transfer of antimicrobial-resistant bacteria between companion animals and humans

During the last fifty years, the number of companion animals has substantially increased to the point that in many regions, most people have regular and intensive contact with companion animals, which are nowadays considered as “family members” enjoying close contact with their owners (Guardabassi et al. 2004; Dotson and Hyatt 2008; Pomba et al. 2017). The anthropomorphization of companion animals has led to changes in the behaviour of owners towards them, with increasing conducts like kissing, licking, sharing food, and sharing beds (Dotson and Hyatt 2008). Considering the shared environment of humans and companion

animals, their close relationship, and the increased frequency of the presence of antimicrobial-resistant bacteria in humans and companion animals, new opportunities are created for interspecies transfer of resistance genes, resistant bacteria or mobile resistance determinants.

The closer contact between owners and companion animals creates opportunities for pathogen interchange through direct and indirect contact (Guardabassi et al. 2004; Damborg et al. 2016; Pomba et al. 2017). Furthermore, during infection, companion animals have and excrete high loads of pathogenic bacteria, which may further favour this transmission (Pomba et al. 2017).

The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals have been reviewed in the European Medicine Agency and its Antimicrobial Working Party reflection paper (Pomba et al. 2017). Pomba et al. (2017) alerted for existence of several antimicrobial resistance microbiological hazards coming from companion animals to humans (Table 4).

Table 4: Microbiological hazards from companion animals to humans identified by EMA (adapted from Pomba et al. 2017).

Antimicrobial-resistant bacteria	Type of Hazard	Source
MRSA	direct hazard[1]	dogs, cats and horses
MRSP	direct hazard	dogs, cats and horses
VRE	indirect hazard[2]	dogs and horses
ESBL-producing Enterobacteriaceae	indirect hazard	dogs, cats and horses
Carbapenem-resistant Gram-negative bacteria	indirect hazard[2]	Dogs and cats
Colistin-resistant *E. coli*	indirect hazard	Dogs and cats

Legend: [1]Low number of cases of human infections originating from companion animals. [2]No human infections originating from companion animals have been reported (Pomba et al. 2017).

The concerns surrounding the role of companion animals in the dissemination of resistant bacteria to humans are strengthened by numerous studies reporting the colonization and/or infection of companion animals with bacteria harboring clinically relevant antimicrobial resistance mechanisms or bacteria belonging to high-risk clonal lineages to humans (Guardabassi et al. 2004; Nicolas-Chanoine et al. 2014; Damborg et al. 2016; Pomba et al. 2017; Grönthal et al. 2018).

Transmission of antimicrobial-resistant bacteria or resistance genes between humans and companion animals (colonized and/or infected) has been reported in different studies over the last years, which implies a potential risk of the transmission of resistance genes by humans via direct contact with their companion animals and vice-versa (Johnson and Clabots 2006;

Johnson et al. 2008; Pomba et al. 2017). Moreover, the detection of MDR bacteria in companion animals is being increasingly reported, posing a difficult veterinary therapeutic challenge and often requiring the use of antimicrobials critically important to humans. With the growing contact between companion animals and humans, the risk of animal-to-human transfer of such bacteria is of concern. These bacteria, isolated from companion animals, may harbour clinically and epidemiologically important resistance mechanisms of human and veterinary relevance such as ESBL, cephalosporinases (AmpC), PBP2a and high-level gentamicin resistance (HLGR) bifunctional enzyme (Marques et al. 2016).

Coagulase-positive staphylococci, such as methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus pseudintermedius* (MRSP), *Staphylococcus intermedius* (MRSI), methicillin-resistant coagulase-negative staphylococci (MR-CoNS) and ESBL/AmpC- or carbapenemase producing-Enterobacterales has been documented to colonize companion animals and humans causing infections in both species (Guardabassi et al. 2004; Vincze et al. 2014; Schmiedel et al. 2014; Misic et al. 2015; Damborg et al. 2016; Ljungquist et al. 2016; Pomba et al. 2017; Walther et al. 2017;Marques et al. 2019a, 2019b, 2019c). These studies also have reporting that companion animals may share antimicrobial-resistant bacteria or resistance genes with household members and show that colonized and/or infected companion animals with bacteria belonging to high important clonal lineages described in humans. Such as, MRSA sequence type (ST) 5 and ST22 (Weese 2010; Couto et al. 2016), *Enterococcus faecium* clonal complex (CC) 17 (Damborg et al. 2009), *E. coli* ST131, ST648 and ST405 (Johnson et al. 2008a, 2008b; Johnson et al. 2009; Johnson et al. 2016a; Marques et al. 2018), *Klebsiella pneumonia* ST11 and ST15 (Marques et al. 2019b) and carbapenemase-producing *A. baumannii* ST2 (Pomba et al. 2014a).

Regarding transmission of carbapenemases-producing bacteria between humans with contact with companion animals, few studies were found. Wang et al. (2017) tested fecal samples from farmers and dogs from the local and *E. coli* strains with the carbapenemase NDM-5 enzyme were shared between humans and dogs. Furthermore, Grönthal et al. (2018) demonstrated that two dogs with SSTI (otitis) shared NDM-5-producing *E. coli* strains with one human living in the same household.

The conclusions achieved indicate that it is necessary to reflect on how antimicrobials are being used and that measures are needed to implement and promote their use in a rational manner. Antimicrobials are frequently administered empirically based on the presence of compatible clinical signs; however, antimicrobial therapy should ideally rely on susceptibility testing of the isolated bacteria. Besides the pharmacokinetic-pharmacodynamic properties, the empiric antimicrobial selection should consider the most likely causative agent as well as its regional susceptibility patterns. Furthermore, according to the World Organisation for Animal

Health veterinarians should adopt strategies aimed at the reduction of antimicrobial resistance (Marques et al. 2016).

1.4 *Escherichia coli*

E. coli was first discovered, as “Bacterium coli commune”, in the gut in 1885 by the German bacteriologist Theodore von Escherich (Escherich 1988). *E. coli* is a member of the genus *Escherichia* which is one of the key genera of the Enterobacteriaceae family. Over the past decades *E. coli* is one of the most-studied bacteria and become one of the best-characterised Enterobacterales.

E. coli is part of the common commensal inhabitant of the gastrointestinal tract of humans and animals. It colonizes the gastrointestinal tract within hours after birth and there is a mutual relationship (symbiotic) between host and bacteria. (Sarowska et al. 2019). This relationship between the bacterium and its host is symbiotic, providing both with several advantages. The *E. coli* strains can be classified according to genetic and clinical criteria into three main groups: commensal *E. coli*, intestinal pathogenic *E. coli* and extraintestinal pathogenic *E. coli* (ExPEC) (Figure 6) (Nicolas-Chanoine et al. 2014; Paulshus et al. 2019; Sarowska et al. 2019). Commensal *E. coli* are in general benign; they coexist with the human host with mutual benefits and do not cause disease. However, it may cause illness if the host is compromised immunologically or if the normal gastrointestinal barriers are breached (Russo and Johnson 2000). Intestinal *E. coli* pathogenic strains cause enteric/diarrhoeal diseases and six different categories have been described: enteropathogenic, enterohaemorrhagic, enterotoxigenic, enteroaggregative, enteroinvasive and diffusely adherent (Nataro and Kaper 1998). ExPEC have maintained the ability to exist in the gut without consequence but have the capacity to disseminate and colonize other host niches causing extra-intestinal diseases including soft-tissue infections, wound infections, neonatal meningitis, sepsis, hospital-acquired pneumonia, osteomyelitis, surgical site infection haemolytic-uremic syndrome and UTIs (Nataro and Kaper 1998; Russo and Johnson 2000; Johnson and Russo 2005; Bélanger et al. 2011; Sarowska et al. 2019).

E. coli causing UTI has been denoted uropathogenic *E. coli* (UPEC) (Russo and Johnson, 2000). *E. coli* strains has an extensive genetic substructure (Chaudhuri and Henderson 2012) and that the substructure of *E. coli* populations differs among distinct geographical regions (Freitag et al. 2005; Walk et al. 2009) and bacterial hosts (Vadnov et al. 2017). Urinary tract infection by *E. coli* is a problem that affects both humans and companion animals over the world (Foxman 2014; Flores-Mireles 2015; Sarowska et al. 2019).

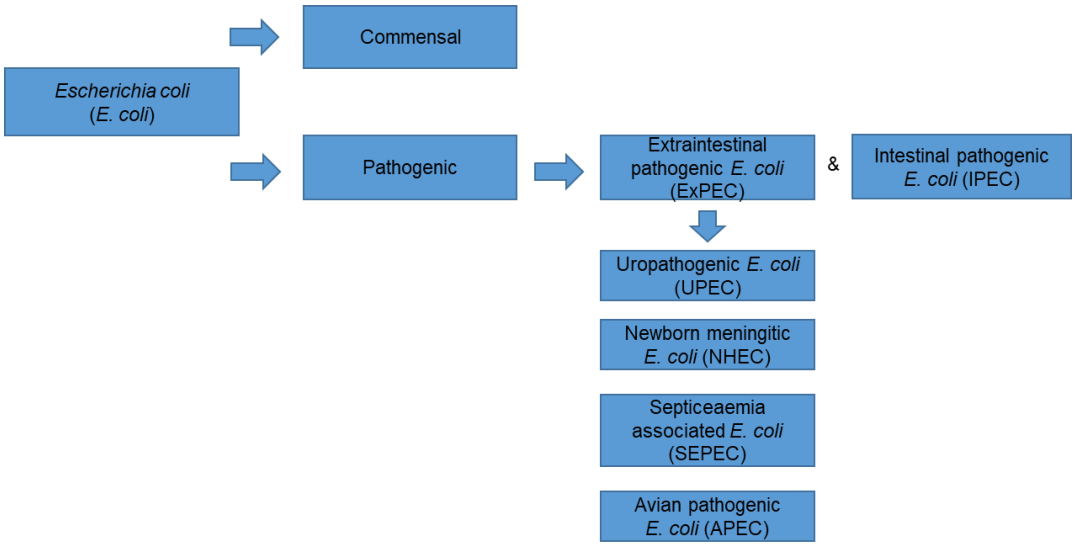


Figure 6: Pathogenic diversity of *Escherichia coli* strains (adapted from Sarowska et al. 2019).

The worldwide burden of these extraintestinal infections is staggering, with hundreds of millions of people affected annually and considerable morbidity and mortality in cases of complication with bacteremia or sepsis syndrome (Nicolas-Chanoine et al. 2014). Different studies around the world have identified several potential reservoirs for the *E. coli* strains that cause most human ExPEC infections, including the human gastrointestinal tract, companion animals, wild animals, food-producing animals, retail meat products, sewage, environmental sources and infections obtained abroad during travelling (Johnson et al. 2001; Ewers et al. 2009; Johnson et al. 2009, Jakobsen et al. 2010a; Bélanger et al. 2011; Manges and Johnson 2015). One of major concern is a possible transmission of virulent and/or resistant *E. coli* between animals and humans. *E. coli* also represents one of a major reservoir of resistance genes that may be responsible for treatment failures in both human and veterinary medicine (Nicolas-Chanoine et al. 2014; Poirel et al. 2018). The prevalence of resistance to first-line oral antimicrobials, such as amoxicillin and amoxicillin-clavulanic acid, fluoroquinolones and trimethoprim-sulfamethoxazole, which are widely used to treat community-acquired *E. coli* infections, has increased over the last 20 years (Guardabassi et al. 2004; Nicolas-Chanoine et al. 2014; Sarowska el al. 2019).

1.5 Plasmids carring ESBLs/AmpC and carbapenemases in *Escherichia coli*

A common vector for the transmission of antimicrobial resistance genes is extra-chromosomal DNA, a circular molecule denominated plasmid. Plasmids are small DNA molecules that naturally exist within bacterial cells and replicate independently from the chromosome (Lee et al. 2018; Partridge et al. 2018; Stohr et al 2019). Furthermore, plasmids have systems which guarantee their autonomous replication and also have mechanisms controlling their copy number and ensuring stable inheritance during cell division (Carattoli 2009).

Plasmids can harbour genes involved in antimicrobial resistance and virulence and are important vectors for horizontal gene transfer (Carattoli 2013; Stohr et al 2019). They contribute to the spread of relevant resistance determinants, promoting horizontal gene transfer among different bacteria and strains, favouring their dissemination among the bacterial population and from region to region (Carattoli et al. 2013; Lee et al. 2018). These plasmids belong to families that are prevalent in naturally occurring bacteria, usually carry multiple physically linked genetic determinants, conferring resistance to different classes of antimicrobials simultaneously (Carattoli 2013). Moreover, the knowledge of the epidemiology of plasmids is important for understanding the evolution and spread of antimicrobial resistance (Stohr et al 2019).

Hedges and Datta (1971) proposed a plasmid classification scheme based on the stability of plasmids during conjugation, this phenomenon have the name of plasmid incompatibility (Datta and Hedges1971; Hedges and Datta 1971; Carattoli 2009). Incompatibility was defined as the inability of two related plasmids to be propagated stably in the same cell line; thus, only compatible plasmids can be rescued in transconjugants (Carattoli 2009). Moreover, plasmids can acquire mobile genetic elements such as transposons and Insertion sequences (IS), which can lead to mobilization of antimicrobial resistance (Alekshun and Levy 2007). Plasmids may carry genes that are responsible for conferring resistance to the major classes of antimicrobials including β-lactams, quinolones, aminoglycosides, tetracyclines, sulphonamides, trimethoprim and chloramphenicol (Caratolli et al. 2009).

The global dissemination of the CTX-M enzymes has been attributed to the association of plasmids carrying *bla*$_{\text{CTX-M}}$ that belong mostly to incompatibility groups IncF, IncN, and IncK with certain insertion sequences (ISs), such as IS*Ecp1* or IS*CR1*, is able to capture and mobilize *bla*$_{\text{CTX-M}}$ genes effectively among the Order Enterobacterales. Insertion sequences elements can also act as strong promoters for the high-level expression of *bla*$_{\text{CTX-M}}$ (D'Andrea et al. 2013; Mathers et al. 2015a; Poirel et al. 2018). Furthermore, analysis of genetic environments of CTX-M enzymes has revealed that the promoter sequence present in the upstream region significantly affects gene expression and dissemination (Sabaté et al. 2002, Munday et al. 2004; Garcia et al. 2005; Oliver et al., 2005; Cantón and Coque 2006; Poirel et al. 2018). Furthermore, the genes encoding ESBL enzymes are usually located in plasmids but can also be found in the chromosomal DNA. The *bla*$_{\text{CTX-M-15}}$ gene has been located mainly on plasmids belonging to the IncF group (Carattoli 2009; Kondratyeva et al. 2020). IncF plasmids are low-copy-number plasmids, often carrying more than one replicon. Moreover, IncF plasmids group carrying the *bla*$_{\text{CTX-M-15}}$ gene are not a homogeneous, because they vary in size (50 to 200 kb), carry the repFII replicon alone or in combination with repFIA or/and repFIB, and can have different antisense RNA sequence variants in the repFII replicon (Hopkins et al. 2006; Carattoli et al. 2008; Carattoli 2009). Furthermore, the IncF plasmids has great versatility of intracellular adaptation by the rapid evolution of the regulatory sequences of the replicons. (Villa et al. 2010). IncF plasmid group are

higher diffused in clinically relevant *E. coli* strains and is the group most frequent in clinical isolates (Villa et al. 2010). IncF plasmids encoding *bla*$_{CTX-M-15}$ have been isolated from both human (Boyd et al. 2004, Hopkins et al. 2006; Karisik et al. 2006; Coque et al. 2008; Kondratyeva et al. 2020) and companion animal (Shaheen et al. 2011; Hou et al. 2012; So et al. 2012; Dahmen et al. 2013; Yang et al. 2015; Kawamura et al. 2017) *E. coli* isolates. Moreover, IncF-like plasmid is also, involved in the dissemination of *bla*$_{CTX-M-14}$ (Woodford et al. 2009; Ho et al. 2012).

IncI1 plasmids were associated with the spread of several other ESBL genes, such, as *bla*$_{CTX-M-1}$ in human and animals *E. coli* isolates (especially food-producing) (Roer et al. 2019; Valcek et al. 2019; Irrang et al. 2018). Yet, for the IncI1 plasmids different sequence types have been described, indicating great variability among the members of the IncI1 family (Caratolli 2009). Besides the IncI1 plasmids, the *bla*$_{CTX-M-1}$ genes was also identified on plasmids belonging to the IncN group (Dolejska et al. 2011; Dolejska et al. 2013; Rodrigues et al. 2013).

Most of the *bla*$_{CMY-2}$ plasmids identified in *E. coli* were associated with the IncA/C group. Such plasmids have been isolated from both from humans and animals, (Carattoli et al. 2012). The second most common replicon type associated with *bla*$_{CMY-2}$ is I1, whereas other replicon types have been reported sporadically (Carattoli 2009). Compared to other pAmpC variants the success of CMY-2 is probably associated with its relationship with insertion sequence IS*Ecp1* (Naseer et al. 2010) which provides the promotor regions that drives high-level expression of *bla*$_{CMY}$ (Nakan et al. 2007), like what has been observed for *bla*$_{CTX-M-15}$. Regarding the emergence and rapid spread of carbapenemases-producing *E. coli* (*bla*$_{OXA-48}$, *bla*$_{NDM-1}$, *bla*$_{KPC-2}$ and *bla*$_{VIM-2}$) is, in part, the consequence of conjugative plasmids (pOXA-48 of IncL/M-type) and also, of mobile genetic elements (Tn*4401* for KPC-2, Tn*1999* for OXA-48, Tn*125* for NDM-1 and Tn*21* associated with integrons for VIM/IMP) (Aubert et al. 2006; Naas et al 2008; Poirel and Nordmann 2014; Zhao and Hu 2015; Peirano and Pitout 2015; Vila et al 2016). Multi locus sequence typing (MLST) analysis of a worldwide human collection of OXA-48 producing *E. coli* isolates has revealed some diversity among the isolates, suggesting the spread of plasmid pOXA-48 in different clones (Potron et al. 2013; Vila et al 2016). Furthermore, pOXA-48 and IncX3 (associated with *bla*$_{OXA-181}$) plasmids were also, detected in companion animals (Pulss et al. 2018; Nigg et al. 2019).

1.6 *Escherichia coli* phylogenetic groups

E. coli can be considered as having mainly a clonal genetic structure and phylogenetic analyses based upon multi locus enzyme electrophoresis (MLEE) and ribotyping have shown the existence of distinct phylogenetic groups within *E. coli* (Ochman and Selander 1984; Herzer et al. 1990; Desjardins et al. 1995). There are four well known phylogenetic groups and these have been nominated A, B1, B2 and D. *E. coli* strains of the four phylogenetic groups differ in their phenotypic and genotypic characteristics and appear to have different ecological niches and

propensity to cause disease. Extra-intestinal pathogenic and commensal *E. coli* typically differ with respect to *E. coli* reference (ECOR) collection phylogenetic group (Sabaté et al. 2006). Commensal *E. coli* are mainly associated with phylogenetic groups A or B1 (with lower number of virulence genes) and intestinal pathogenic *E. coli* with phylogenetic groups A, B1 or D (Picard et al. 1999; Russo and Johnson 2000; Johnson et al. 2001; Johnson and Russo 2002; Escobar-Páramo et al. 2004; Sabaté et al. 2006). Yet, ExPEC including UPEC have shown to belong mainly to phylogenetic group B2 and to group D (harbouring a high number of virulence genes) (Picard et al. 1999; Johnson et al. 2005; Escobar-Páramo et al. 2004; Moreno et al. 2008). However, MLEE or ribotyping are complex and time-consuming techniques. So, Clermont et al. (2000) implemented a rapid phylogenetic grouping technique based on triplex PCR to type and subtype commensal and pathogenic *E. coli*, based the combination of the following genetic markers: chuA, yjaA (encoding hypothetical proteins) and the DNA fragment TspE4.C2 (encoding a putative lipase esterase) and regarding the presence/absence of these three amplicons, an *E. coli* strain could be classified into one of the main phylogenetic groups (Clermont et al. 2000; Doumith et al. 2012). However, Escobar-Páramo et al. (2006) in order to increase the discrimination power of *E. coli* population analyses, it has been proposed the use of subgroups A_0, A_1, B_1, B_{22}, B_{23}, D_1 and D_2, that are determined by the combination of the genetic markers (Clermont et al. 2000; Escobar-Páramo et al. 2006). However, subsequently, on the basis of MLST and complete genome data, additional *E. coli* phylogenetic groups were recognized (Walk et al. 2009; Luo et al. 2011). So, the number of defined phylogenetic groups went up to 8 (A, B1, B2, C, D, E, F that belong to *E. coli sensu stricto*, and the eighth-the *Escherichia* cryptic clade I) (Walk et al. 2009; Luo et al. 2011). In 2012 Doumith et al. implemented a new multiplex PCR for rapid assignment of the four major *E. coli* phylogenetic groups (Doumith et al. 2012) and in 2013 Clermont et al added an additional gene target, *arp*A, to those three candidate markers and made a quadruplex PCR to classify an *E. coli* strain into one of the phylogenetic groups A, B1, B2, C, D, E, F, and clade I (Clermont et al. 2013). Furthermore, phylogenetic group E and group F are related to phylogenetic group D and group B2, respectively and clones of *E. coli* strains, which are genetically diverse but phenotypically indistinguishable, have been assigned to cryptic clade I (Walk et al 2009; Köhler and Dobrindt 2011; Luo et al. 2011; Sarowska et al. 2019).

1.7 *Escherichia coli* pathogenicity-associated islands markers

Pathogenicity islands (PAIs) are large blocks of chromosomal DNA (>30 kb), unstable regions of chromosomally located DNA, that can be inserted or deleted from the genome; can be characterized by the different G+C content from the rest of the genome and in different codon usage; are often associated with transfer RNA (tRNA) genes like pheU, pheV, selC and leuX5; often carry cryptic or functional genes containing mobile genetic elements such as transposons, integrases and insertion sequences (IS) elements or parts of these elements (Hacker et al. 1997;

Hacker and Kaper 2000; Sabaté et al. 2006). PAIs normally represent unstable DNA regions. Deletions of PAIs may occur via the direct repeats (DRs) at their ends or via IS elements or other homologous sequences located on PAIs (Hacker and Kaper 2000). PAIs contain bacterial virulence genes (such as: genes for P fimbriae, S fimbriae, cytotoxic necrotizing factor 1, yersiniabactin) mostly located on bacterial chromosome but also in plasmids or bacteriophages (Hacker et al. 1997; Hacker and Kaper 2000; Oelschlaeger et al. 2002; Gal-Mor and Finlay. 2006). PAIs and their associated virulence genes spread among bacterial populations by horizontal transfer and supply a virulence benefit regarding the adaptation to niches incapable to be colonize by commensal bacteria (Hacker et al 1997; Oelschlaeger et al. 2002; Lloyd et al. 2009; Sarowska et al. 2019).

Several PAIs have previously been identified in UPEC strains such as *E. coli* 536, *E. coli* J96, *E. coli* CFT073. PAIs I to IV from strain 536 encode a range of virulence factors, including P fimbriae, P-related fimbriae, α-hemolysin, S-fimbriae and yersiniabactin siderophore system. PAIs I_{J96} and II_{J96} encode P fimbriae, P-related fimbriae and α-hemolysin. PAIs I_{CFT073} and II_{CFT073} encode P fimbriae, α-hemolysin and aerobactin (Sabaté et al. 2006). These UPEC PAIs have been also detected in commensal isolates from fecal origin (Johnson and Russo 2002; Sabaté et al. 2006). In 2000 Kurazono described a putative pathogenicity island (PAI) containing the gene encoding uropathogenic specific protein (usp) in UPEC strain Z42 and three small open reading frames (orfU1, orfU2, and orfU3) encoding 98, 97, and 96 amino acid proteins located downstream of the *usp* gene (Kurazono 2000) It has been demonstrated that the plasmid containing the *usp* gene enhances the infectivity of host *E. coli* strains in a mouse pyelonephritis model suggesting that the *usp* gene contributes to the pathogenesis of urinary tract infection (UTI) (Yamamoto 2001).

Moreover, ExPEC strains often contain multiple PAIs and can have a different combination of virulence factors (VFs) (Johnson and Russo 2005). Furthermore, the same VF can be encoded by different PAIs. In commensal isolates these DNA segments can act as fitness islands or ecological islands rather than as PAIs (Hacker and Kaper 2000). Moreover, PAIs may also carry genes encoding factors that confer antimicrobial resistance substances (Hacker and Carniel 2001). The characteristics of the pathogenicity islands and the function encoded are presented in Table 5.

1.8 Virulence factors of Extraintestinal Pathogenic *Escherichia coli* and/or Uropathogenic *Escherichia coli*

Virulence refers to the ability of a pathogen to cause disease. Virulence factors (VFs) are specific properties that enable organisms to overcome host defenses and cause disease (Johnson 1991). The virulence potential of ExPEC depends on the various extraintestinal

Table 5: The pathogenicity islands and the functions encoded (adapted from Sarowska 2019).

Pathogenicity islands	Products
I_{CFT073}	P fimbriae, α-hemolysin and aerobactin
II_{CFT073}	P fimbriae, α-hemolysin and aerobactin
I_{536}	α-Hemolysin, F-17-like fimbriae, CS12-like fimbriae
II_{536}	Hek adhesin, P- related fimbriae, α-Hemolysin, hemagglutinin-like adhesion
III_{536}	S fimbriae, salmochelin, HmuR-like heme receptor, Sat toxin, Tsh-like hemoglobin protease, antigen 43
IV_{536}	Yersiniabactin siderophore system
I_{J96}	α-Hemolysin, Prs fimbriae, cytotoxic, necrotizing factor
II_{J96}	α-Hemolysin, Prs fimbriae, cytotoxic, necrotizing factor

virulence-associated factors in bacteria–host interactions, rather than a simple mechanism (Picard et al 1999). Commensal and pathogenic bacteria typically differ with respect to phylogenetic background and VFs profiles (Johnson and Stell 2000). These VFs can be grouped by functional category (Table 6). Characteristic ExPEC virulence factors include various adhesins (P, S and type 1 fimbriae among others), iron acquisition and utilization systems (aerobactin, salmochelin and yersinianbactin siderophores), protectins (structural components of the bacterial outer membrane), toxins (hemolysin, cytotoxic necrosis factor, autotransporter toxins), and biofilm formation factor (curli fimbriae, antigen 43 and UpaG), extracellular lipopolysaccharides, polysaccharide capsules, and serum resistance (Sarowska et al. 2019). These virulence factors facilitate colonization and invasion of the host, as well as avoidance or disruption of host defense mechanisms (Sarowska et al 2019). ExPEC strains were defined by Johnson et al. (2003) as *E. coli* isolates containing two or more of the following virulence markers: *papA/papC*, *sfa*/*foc*, *afa*/*dra*, *kps*MTII, and *iut*A. Virulence genes (VGs) responsible for pathogenicity are usually encoded on pathogenicity islands (PAIs), plasmids, and other mobile genetic elements, and can thus be transmitted via horizontal gene transfer between various *E. coli* strains (Köhler and Dobrint 2011). Horizontal transfer can occur with PAIs (Hacker et al 1997; Hacker and Kaper 2000; Oelschaeger et al. 2002; Gal-Mor and Finlay 2006). Futhermore, virulence genes may be exclusively chromosomal, such as *pap* and *hly* (encoding P fimbriae and hemolysin, respectively) or occurring in either location, such as afa/dra (coding for Dr antigen-specific adhesion) (Johnson 2003).

Table 6 - Virulence factors of Extraintestinal Pathogenic *Escherichia coli*or Uropthogenic *Escherichia coli* (adapted from Sarowska et al. 2019).

Functional category	Name	Gene	Function	Reference
Adhesin	Dr binding adhesin	*Afa/draBC*	Adhesin, association with cystitis and pylenonephritis, invasion of urothelium	Johnson 1991; Mulvey 2002; Sarowska et al. 2019
	Blood group M fimbria	*bmaE*	Adhesin	Johnson 1991
	Type 1 fimbria	*fimH*	Adhesin, factor of colonization in extraintestinal infections, mediates binding to urothelium and invasion, role in IBC formation and biofilm formation	Johnson 1991; Mulvey et al; 1998; Wright et al. 2007; Sarowska et al. 2019
	F1C fimbria	*focG*	Adhesion to renal epithelial cells and endothelial cells of the bladder and kidneys	Mulvey et al. 2002
	Iron-regulated gene A homologue adhesion	*iha*	Adhesin, siderophore function	Johnson et al 2000; Sarowska et al. 2019
	Intimin-like factor adherence *E. coli* (FdeC)	*eaeH*	Adhesin, adhesion to mammalian cells and extracellular matrix	Nesta et al. 2012
	G fimbria	*gafD*	Adhesin	Johnson 1991; Johnson and Stell 2000
	P fimbria	*papAH*	Adhesin, mediate binding to urothelium, association with pylenonephritis	Mulvey 2002; Sarowska et al. 2019
	S fimbria/F1C fimbria	*sfa/focDE*	Adhesin, association with cystitis and pylenonephritis	Mulvey 2002; Sarowska et al. 2019
Biofilm related	Antigen 43	*agn43 (flu)*	Adhesin, autotransporter, aggregation and biofilm related	Ulett et al. 2007; van der Woude and Henderson 2008; Sarowska et al. 2019
	Antigen 43, allele a CFT073	*agn43*a$_{CFT073}$	Adhesin, autotransporter, aggregation and biofilm related	Ulett et al. 2007; van der Woude and Henderson 2008
	Antigen 43, allele b CFT073	*agn43*b$_{CFT073}$	Adhesin, autotransporter, aggregation and biofilm related	Ulett et al. 2007; van der Woude and Henderson 2008
	Antigen 43, allele K12	*agn43*a$_{K12}$	Adhesin, autotransporter, aggregation and biofilm related	Ulett et al. 2007; van der Woude and Henderson 2008

	Curli fibers	*cgsBAC/ cgsDEFG*	Adhesin, enable biofilm formation	Vidal et al. 1998; Chapman et al. 2002; Sarowska et al. 2019
	Trimeric autotransporter adhesin (UpaG)	*upaG*	Adhesin, aggregation and biofilm formation	Valle et al. 2008
Iron uptake	Heme transport protein	*chuA*	Enable using of Fe from hemoglobin in the host system	Clermont et al. 2000; Sarowska et al. 2019
	SitABCD operon	*sitABCD*	Iron acquisitin, periplasm iron binding protein	Sarowska et al. 2019
	Yersinian siderophore receptor	*fyuA*	Phenolate siderophore,uptake of ferric iron	Henderson et al. 2009; Sarowska et al. 2019
	Aerobactin	*iucABCD*	A mixed-type siderophore, acquisition of Fe2 + / 3 + in the host system	Sarowska et al. 2019
	Salmochelin siderophore receptor	*iroN*	Catecholate siderophore receptor, use of Fe ions obtained from the body host	Johnson et al. 2000b; Henderson et al. 2009 Sarowska et al. 2019
	Iron-regulated element	*ireA*	Siderophore, uptake of ferric iron	Russo et al. 2001; Henderson et al. 2009
	Aerobactin siderophore receptor	*iutA*	Siderophore, uptake of ferric iron	Henderson et al. 2009; Sarowska et al. 2019
	CjrABC	*cjrABC*	Iron acquision, may contribute to urovirulence	Mao et al. 2012
Protectins/serum resistance	Increased serum survival	*iss*	Outer membrane protein, resistance to serum bactericidal activity	Johnson 1991; Johnson and Stell 2000
	Group II capsule	*KpsM II*	Protect against phagocytosis, opsonisation and lysis	Johnson 1991; Rama et al. 2005
	Group II capsule K2	*KpsM II K2*	Protect against phagocytosis, opsonisation and lysis	Johnson 1991; Rama et al. 2005
	Group III capsule	*KpsM III*	Protect against phagocytosis, opsonisation and lysis	Johnson 1991
	Outer membrane protein	*omp*	Enable intracellular survival, evasion from the body's defense	Sarowska et al. 2019
	Serum resistant	*traT*	Outer membrane protein, resistance to serum bactericidal activity	Johnson 1991; Johnson and Stell 2000

Toxins	Alpha hemolisin	*hlyA*	Cell lysis, modulation of host signal pathways, tissue injury, exfoliation of urothelium.	Johnson 1991; Michaud et al. 2017; Sarowska et al. 2019
	Cytolethal distending toxin	*cdtB*	Create abnormalities in host cell function or morphology, cell cycle arrest or lysis	Johnson and Stell 2000 Fox et al. 2004; Ge et al. 2007; McAuley et al. 2007.
	Cytotoxic necrotizing factor 1	*cnf1*	Engaging in cell necrosis	Michaud et al. 2017; Sarowska et al. 2019
	Serin protease autotransporter	*pic*	Protease involved in colonization, degrades mucins, facilitates colonization epithelium, damages of the cell membrane;	Abreu et al. 2015; Abreu et al.2016; Sarowska et al. 2019
	Vacuolating autotransporter toxin	*vat*	Proteolytic toxin, induces host cell vacuolization	Parham et al. 2005; Nichols et al. 2016; Sarowska et al. 2019
	Secreted autotransporter toxin	*sat*	Proteolytic toxin, create abnormalities in host cell function or morphology, cell cycle arrest or lysis	Guyer et al 2000; Guyer et al 2002; Sarowska et al. 2019
	Plasmid encoded enterotoxin	*senB*	Its role is not yet clear	Cusumano et al. 2010; Mao et al. 2012
Miscellaneous	Invasion of brain endothelium	*ibeA*	Invasion of endothelium	Johnson and Stell 2000
	Arginine deiminase operon	*arcACBDR*	Increase fitness	Billard-Pomares et al. 2019
	Pathogenicity-associated island marker of CT073	*malX*	Maltose- and glucose-specific component IIa of a phosphoenolpuryvate-dependent phosphotransferase system. Encoding different VF, marker of PAIs	Johnson and Stell 2000; Sarowska et al. 2019
	Uropathogenic specific protein	*usp*	Putative bacteriocin	Parret and De Mot 2002; Zaw et al 2013

1.8.1 Adhesins

Bacterial adhesion ensures attachment to host cell surfaces and is the crucial step for the establishment of infection (Rangel et al. 2013). Bacterial adhesion by fimbrial adhesins contribute to virulence by directly triggering host and bacterial cell signalling pathways, which promove the delivery of bacterial products to host tissues and promotes bacterial invasion (Bien et al. 2012).

The primary bacterial adherence factors are filamentous adhesive organelles known as fimbriae (pili), which are presented on bacterial surface or as afimbrial anchored within the bacterial outer membrane (Bower et al. 2005; Rangel et al. 2013). Fimbriae include type I fimbriae and P fimbriae which are present in about 90% and in 40-60% of the *E. coli* strains, respectively (Blanco et al. 1997; Miyazaki et al. 2002). Type 1 fimbriae is encoded by *fim* gene cluster and is highly conserved and commonly expressed by commensal and pathogenic *E. coli* strains and have affinity for structures containing mannose residues (classified as type 1) (Johnson et al. 1991; Rangel et al. 2013; Sarowska et al. 2019). Furthermore, mannose resistant fimbriae are classified as type 2 (P, S and Dr fimbriae) (Table 6) (Källenius et al. 1981; Domingue et al. 1985; Connell et al.1996; Rangel et al. 2013; Sarowska et al. 2019).

Type 1 fimbriae mediate the adhesion and also the invasion and internalization of uroepithelial cells and trigger Toll-like receptor 4 (TLR4) signalling pathway (Pizarro-Cerdá and Cossart 2006; Ragnarsdóttir et al. 2011).

FimB and *fimE* are responsible for controlling the expression of type 1 fimbriae. Moreover, three other genes, such, *fimF, fimG* and *fimH,* are involved in the adhesive property and longitudinal regulation (Eto et al. 2007; Rangel et al. 2013; Sarowska et al. 2019). FimH adhesin, which is formed from FimA protein subunits, binds to uroplakin 1A receptor (UP1a) of bladder epithelial cells, allowing invasion and formation of biofilm-like intracellular structures (Eto et al. 2007; Rangel et al. 2013; Sarowska et al. 2019).

Regarding mannose-resistant fimbriae, P fimbriae genes are commonly found in UPEC strains and are encoded by pyelonephritis-associated pili genes cluster (*pap* gene cluster), which are encoded by eleven genes in the *pap* genes cluster (Bien et al. 2012; Sarowska et al. 2019). Moreover, P fimbriae mediates attachement through PapG adhesin and occurs in different molecular variants: PapGI, PapGII, PapGIII, with PapG III allele associated with bladder inflammation in women and children (Johnson et al 2000; Wiles et al 2008; Sarowska et al. 2019), and PapGII related to human bacteremia and pyelonephritis (Johnson et al. 2000; Lane and Mobley 2007; Bien et al. 2012; Sarowska et al. 2019).

P fimbriae and type 1 fimbriae help bacteria bind and invade uroepithelial cells in the adverse conditions of the urinary tract (Bien et al. 2012; Sarowska et al. 2019).

Regarding F1C fimbriae (*foc*), this fimbriae can bind β-GalNac-1, 4b-Gal residues on glycolipids expressed by epithelial cells of distal tubules and cells of the collecting ducts of the kidney, as well as by endothelial cells of the bladder and kidneys (Mulvey et al. 2002; Sarowska

et al. 2019). F1C fimbriae are genetically associated with S-type fimbriae (*sfa*) (Johnson 1991; Bien et al 2012; Sarowska et al. 2019). These two adhesins show a high degree of homology, but they differ in receptor specificity (Bien et al. 2012; Sarowska et al. 2019). S fimbriae adhesins recognize receptors containing sialic acid sugar moieties and have the capacity to agglutinate human erythrocytes (Johnson 1991). The *sfa* gene cluster consists of six subunits (*sfaA*, *B*, *C*, *D*, *G*, *H* and *S*) and the *sfaA* gene have information about the major subunit, and the *sfaS* gene have information about specific adhesion, which binds to the α-sialyl-(2,3)-β-Gal receptor in the renal tubular epithelial cells, renal glomeruli, or vascular epithelium (Bien et al. 2012; Sarowska et al. 2019). SfaS adhesin produced by the S fimbrial adhesion (*sfa*) mediates interactions with sialic acid receptors on renal epithelial and endothelial cells. Futhermore, S fimbriae allow invasion of pathogens to host tissues, and are often detected in strains responsible for ascending UTIs, including pyelonephritis (Bien et al. 2012; Sarowska et al. 2019) (Table 6).

Other important group of superficial virulence factors, which are more prevalent among pyelonephritis and cystitis *E. coli* strains is the Afa/Dr family of adhesins, which contains both fimbrial adhesins (Frömmel et al. 2013) (Table 6). The *afa-1* operon was the first determinant to be identified that encodes for afimbrial adhesins (AFA-I) (Labigne-Roussel et al. 1984; Servin 2005). However, since then more genes of *afa* operon have been described, *afa-2*, *afa-3* and *afa-4* which encode for AFA-II, AFA-III and AFA-IV afimbrial adhesins, respectively (Servin 2005). The Dr adhesin family is composed of fimbiral and afimbrial structures on the surface of *E. coli* that bind to the Dr blood group antigen (Nowicki et al. 1990), a portion of the decay-acceleration factor (DAF), which is a membrane protein that prevents cell lysis by complement and are expressed on the surface of erythrocytes and cells of other tissues, such as the epithelium of the urinary tract (Nowicki et al. 1988; Nowicki et al. 1993; Servin 2005). Moreover, Adhesin Dr family has two other receptors (Westerlund et al. 1989; Nowicki et al. 1990; Servin 2005). One of them is the type IV collagen on basement membranes of kidneys (Westerlund et al. 1989; Nowicki et al. 1990; Servin 2005), Bowman´s capsule and bladder epithelium (Nowicki et al. 1990; Goluszko et al. 1997; Van Loy et al. 2002) and the other important receptor is the carcinoembryonic antigen related to cell adhesion molecules, which serves as a signal receptor regulating physiological changes and is related to the cell adhesion (Servin 2005). Adhesin Dr family encoding *dra* operon (dra*A*, *B*, *C*, *D* and *E*) is required for expression of the mannose resistant haemagglutinin phenotype (Goluszko et al 1997; Servin 2005). Furthermore, *afa* and *dra* operons have very similiar genetic organization and are similar at DNA level. dra*A*, *B*, *C*, *D* (*afaA*, *B*, *C*, *D*) genes encode helper proteins and dra*E*/afa*E* determine the expression of genetic information and the production of adhesins (Servin 2005).

E. coli assemble adhesive amyloid fibers termed curli at the bacterial cell surface that are involved in biofilm formation (Vidal et al. 1998; Chapman et al. 2002; Sarowska et al. 2019) and mediate binding to a variety of host proteins (Sjöbring et al. 1994; Ben Nasr et al. 1996). Biofilms

within the host are implicated in serious and persistent infectious diseases, including UTI chronic otitis media and cystic fibrosis (Donlan and Costerton 2002; Parsek and Singh 2003).

Curli are thin coiled fibers, expressed on the surface of *E. coli* that bind some matrix and plasma proteins such as, fibronectin, laminin, plasminogen, tissue plasminogen activator, and H-kininogen and proteins of the fibrinolytic and contact-phase systems (Sjöbring et al. 1994; Olsén et al. 1998). This capacity maybe facilitates the adaptation of curli-expressing bacteria to different niches in the infected host (Olsén et al. 1998). It has been shown that *E. coli* curli fibres in human plasma absorbs plasminogen and tissue plasminogen activator, leading to the formation of proteolytically active plasmin which may promote bacterial spreading through tissue degradation (Olsén et al. 1998; Antão et al. 2009). Moreover, curli fimbriae in *E. coli* consist of polymers of a single 15-kDa protein and are encoded on the csg (curlin subunit gene) gene cluster (Olsén et al. 1993; Gophna et al. 2001). The curli fibres requires expression of two differently transcribed operons (Olsén et al. 1993; Olsén et al. 1998), one which encodes the *csgB*, *csgA* and *csgC* genes, and a second which encodes *csgD*, c*sgE* and *csgG* genes and are expressed by pathogenic and non-pathogenic *E. coli* strains (Hammar et al. 1995; Gophna et al. 2001; Antão et al. 2009). Curli fibres involves extracellular self-assembly of the subunit *csgA*, dependent on a specific nucleator protein *csgB*. *CsgD* is a transcriptional activator essential for expression of the two curli fibre operons, while *csgG* is an outer membrane lipoprotein involved in extracellular stabilization of *csgA* and *csgB* (Gophna et al. 2001; Antão et al. 2009). The expression of genes coding for curli is complex and involves some elements, such as H-NS, RpoS and OmpR and MlrA, which are involved in a reduction in the expression of curli fibres and in production and extracellular matrix formation (Gophna et al. 2001; Antão et al. 2009).

Another group of adhesins associated with urovirulence is represented by the autotransporter (AT) subgroups of proteins, which represent the largest group of outer-membrane and secreted proteins in Gram-negative bacteria, such as antigen 43 (Ag43) (Table 6) (Heras et al. 2014). AT proteins are defined by a specific domain architecture that comprises an N-terminal signal sequence that directs secretion of the protein across the inner membrane via the general secretory pathway, a passenger (α^{43}) domain that is either anchored to the cell surface or released into the external milieu and determines the functional characteristics of the protein, and a a C-terminal β-barrel domain that forms an integral outer membrane protein (β^{43}) (Ullet et al. 2007; van der Woude and Henderson 2008; Heras et al. 2014).

Ag43 protein is produced as a 1,039-amino acid preprotein incorporating an N-terminal signal peptide that directs translocation across the cytoplasmic membrane into the periplasm (Heras et al. 2014). The Ag43 is associated with cell aggregation and with biofilm formation (Danese et al. 2000; Klemm et al. 2004; Ullet et al. 2007; De Luna et al. 2008; Sarowska et al. 2019). Ag43 (encoded by the *flu* gene) is a self-recognizing AT adhesin that confers characteristic surface properties on host cells, such as autoaggregation and a frizzy colony morphology (Ulett

et al. 2007). Furthermore, Ag43 is found in most *E. coli* pathotypes, including uropathogenic UPEC strains (Roche et al. 2001; Ulett et al. 2007; Heras et al. 2014). This protein is expressed on the surface of UPEC cells located within intracellular biofilm-like bacterial pods in the bladder epithelium, indicating that it may contribute to survival and persistence during prolonged infection (Ullet et al. 2007). Moreover, Ulett et al. (2007) addressed the role of the two agn43 alleles in UPEC isolate CFT073 and both are located on pathogenicity islands. The reference UPEC strain CFT073 contains two Ag43 variants (Ag43a and Ag43b) that share 85% sequence identity in the functional α-domain (Ulett et al. 2007; van der Woude and Henderson 2008). Futhermore, the predicted alpha-domain of their protein products (Ag43a and Ag43b) shares 81.7 and 78.4% amino acid identity, respectively, with the *E. coli* K12 Ag43 alpha-domain (Ag43c) (Ulett et al. 2007; van der Woude and Henderson 2008). However, only Ag43a mediates strong aggregation and biofilm formation and long-term colonization of the mouse urinary tract (Ulett et al. 2007).

A group of nonfimbrial adhesins that have the capacity to form stable trimeric structures (Trimeric AT proteins) on the bacterial cell surface has been described in *E. coli* and in another gram-negative bacteria (Roggenkamp et al. 2003; Surana et al. 2004; Valle et al. 2008). However, in *E. coli* few trimeric AT proteins have been characterized, including UpaG from CFT073, which promotes cell-to-cell aggregation, biofilm formation and adhesion to fibronectin, laminin and bladder epithelial cells (Valle et al. 2008). UpaG adhesin is characterized by a membrane-anchored C-terminal domain that forms a trimeric β-barrel pore and facilitates the translocation of a passenger domain (consisting of an extended stalk and an N-terminal head) to the bacterial cell surface via the type V secretion pathway (Valle et al. 2008).

The virulence factors, such as curli fimbers, Ag43 and UpaG, which promote cell aggregation and especially the production of biofilms, are importante to increased resistance to antimicrobial agentes (Costerton et al. 1999; Ito et al. 2009; Mittal et al. 2015; Sharma et al. 2016).

1.8.2 Extracellular polysaccharides

Extracellular polysaccharides, such as lipopolysaccharide (LPS) and capsule (K antigen), contribute to the virulence of many bacterial pathogens by providing resistance to phagocytosis and protecting against complement-mediated killing, which is often measured as serum resistance (Burns and Hull 1998; Burns and Hull 1999). LPS consists of the highly conserved lipid A-core and repeating O antigen subunits that differ greatly between strains based on the sugar residues and their linkage patterns within the repeating subunits (Sarkar et al. 2014). The lipid A-core and the O antigen subunits are assembled in separate pathways that come together for ligation at the inner membrane (Sarkar et al. 2014). For *E. coli*, at least 80 distinct polysaccharide capsules have been identified, and they have been divided into four major groups based on biochemical and genetic criteria (Whitfield 1999; Sarkar et al. 2014). Group 2 and 3

capsules are associated with ExPEC and protect against phagocytosis, opsonisation and lysis, thereby contributing to extraintestinal virulence (Johnson 1991; Johnson and O'Bryan 2004; Rama 2005). Furthermore, the group 2 (*kpsMTII*) capsular polysaccharides have been associated with UPEC strains (Johnson 1991; Roberts 1996; Rama 2005). The *kpsMTII* gene encodes proteins required for polymer translocation from its site of synthesis to the cell surface (Sarowska et al. 2019).

1.8.3 Siderophores

Iron is essential for microorganisms growth, such as bacteria and is involved in a variety of biological processes (Krewulak and Vogel 2008; Garénaux et al. 2011). Iron, as an electron carrier, plays a key role in basic cellular processes such as reduction of oxygen for synthesis of adenosine triphosphate (ATP), DNA replication, oxygen transport, gene regulation and peroxide reduction (Krewulak and Vogel 2008; Garénaux et al. 2011). Moreover, iron is built into the protein structure as a prosthetic group (Ratledge et al. 2000; Caza et al. 2011). To properly promote metabolic processes, share and display pathogenic properties, bacteria need access to Fe (Skaar 2010). However, in the mammalian host, free iron concentrations are very low and therefore bacteria have developed some strategies for stealing iron from the host (Braun 2001; Wiles et al. 2008). Bacteria developed mechanisms that enable them to acquire iron in the host body, thus survive and cause infection (Cherayil 2011). Some acquired iron mechanisms are from lactoferrin of lymph and mucosal infections, ferritin present in cells and heme of hemoglobin and myoglobin present in erythrocytes and serous transferrin (Braun 2001; Doherty 2007; Garénaux et al. 2011). Moreover, it has been demonstrated by Lau et al. (2016) that ExPEC strains have membrane pumps that transfer Fe to the cell interior, such as the FeoAB pump detected in commensal and pathogenic *E. coli* (Lau et al. 2016), SitABCD transporter (ABC type transporter, ATP-binding cassette) (Sabri et al. 2008), and Hma and ChuA transporters that enable Fe uptake directly from extracellular heme (Garénaux et al. 2011).

The iron uptake systems can be divided into three main categories: heme acquisition systems, siderophore-based systems and receptor-mediated iron acquisition from host proteins, such as transferrin/lactoferrin receptors (Krewulak and Vogel 2008). These iron uptake pathways involve an outer membrane receptor, a periplasmic binding protein (PBP) and an inner membrane ATP-binding cassete transporter (Krewulak and Vogel 2008). In the heme acquisition systems, iron can be directly scavenged from ferritin and hemoglobin through outer membrane transport heme acquisition systems, such as, Hma and Chu (Garcia et al. 2011; Garénaux et al. 2011; Gao et al. 2012). Regarding siderophore-based systems, siderophores (iron carriers) are secondary metabolites, with their primary function being to assist in capturing iron to maintain bacterial growth and development (Su et al. 2016) and are low molecular weight molecules with

high affinity and specific for ferric iron (Andrews et al. 2003), capture iron from environment and solubilize iron (Fe^{3+}) prior to transport (Andrews et al. 2003; Wiles et al. 2008).

Siderophores can be divided into different classes accordingly with their functional groups as catecholate (enterobactin (Ent)); phenolates, hydroxamic acids (aerobactin (IutA)), α-hydroxycarboxylates, ferrienterobactin receptor (FepA), dihidroxy-benzylserine (Cir and Fiu), ferrichrome (FhuA), coprogen (FhuE), ferrioxamine B (FhuF) and citrate (FecA) (Guerinot et al. 1994; Sarowska et al. 2019). Moreover, some siderophores can increase ExPEC virulence, such as, enterobactin and salmochelin (catecholate siderophores), yersiniabactin (phenolate siderophore), and aerobactin (a mixed type siderophore) (Sarowska et al. 2019).

Salmochelin is encoded by the *iroBCDEN* gene cluster located on ColV or ColBM virulence plasmids or identified on PAIs (Bister et al. 2004; Johnson et al. 2006; Sarowska et al. 2019). Furthermore, *IroB* is the gene with glycosyltransferase activity for salmochelin production, which leads to glycosylation of enterobactin that changes its properties from strongly hydrophobic to hydrophilic and this change may contribute to the virulence of ExPEC (Dobrindt et al. 2001; Hantke et al. 2003). The *iroN* gene is an ExPEC salmochelin marker and an important virulence gene in pathogenic ExPEC strains (Olesen et al. 2013).

ExPEC may include another type of siderophore, such as yersiniabactin, this siderophore contributes to the pathogenicity of UPEC, especially during colonization of the urinary tract and may protect bacterial cells against host immune response (Fetherston et al. 2010; Caza et al. 2011; Garénaux et al. 2011). Yersiniabactin (Ybt) is a mixed-type siderophore -dependent iron transport system that is encoded in a chromosomal high-pathogenicity Island (HPI), that containing genes for its biosynthesis, transport, and regulation (Chaturvedi et al. 2012). It may have additional functions such as a chelator of additional metals, including copper and zinc (Chaturvedi et al. 2012). Moreover, UPEC strains appear to use yersiniabactin's copper-binding properties as a mechanism to resist copper toxicity (Bobrov et al. 2014; Holden and Bachman 2015). Some epidemiological studies have suggested that strains of *E. coli* that progress from bladder infection to the kidney are more carriers of the *fyu* gene (Snyder et al. 2004; Mabbet et al. 2009).

Aerobactin is another siderophore characteristic for ExPEC and this siderophore is also encoded by ColV and ColBM virulence plasmids of ExPEC (Johnson et al. 2006; Garénaux et al 2011). Furthermore, *E. coli* have different combinations of iron acquisition systems and the major advantages of this in UPEC are likely to reflect the ability to successfully compete for iron against the host and other bacteria (Watts et al. 2012).

1.8.4 Toxins

Toxins play an important role during an infection as they contribute to the spreading of the bacteria in tissues, induce an inflammatory response and increase cytotoxicity (Vila et al.

2016; Swaroska et al. 2019). These virulence factors were often used to classify ExPEC isolates (Mars et al. 2005). The most frequently detected genes encoding toxins in ExPEC, include: *hlyA (*alfa-hemolysin*)*, *cnf1* (cytotoxic necrotizing factor 1), *sat* (secreted autotransporter toxin), pic (protease involved in colonization), *vat* (vacuolating autotransporter protein), *cdtB* (cytolethal distending factor), and *senB* (Enterotoxin Tie protein) (Table 6) (Johnson 1991; Mao et al. 2012; Vila et al 2016; Sawroska et al. 2019).

The alfa-hemolysin (HlyA), is cytotoxic to a wide range of cells and causes serious tissue damage during UTIs (Sawroska et al. 2019). The hlyA gene is located in the operon *hly*CABD and this can be located in plasmids or on the chromosome (Johnson 1991). HlyA intoxication also stimulated caspase activation, which occurred independently of effects on host serine proteases. HlyA-induced proteolysis of host proteins likely allows UPEC to not only modulate epithelial cell functions, but also disable macrophages and suppress inflammatory responses. (Sawaroska et al. 2019). Moreover, HlyA alters the cytoskeleton of urothelial cells resulting in shedding of bladder urothelium and disruption of urothelial barrier function, induce kidney inflammation and injury and is an important virulence factor in pyelonephritis (Michaud et al. 2017; Sawaroska et al. 2019). HlyA is expressed in 40% of cystitis and about 49% of pylelonephritis human clinical isolates (Yamamoto et al. 1995; Michaud et al. 2017). This virulence factor is produced in the inactive form of pro-HlyA and undergoes a maturation process through HlyC (acetyltransferase) (Johnson 1991). Furthermore, in the gut, HlyA mediates the formation of focal leaks within colonic epithelial cells and can thereby promote the paracellular translocation of bacteria (Sawaroska et al. 2019).

The protein toxin Cytotoxic Necrotizing Factor 1 (CNF1) is an important virulence factor of pathogenic *E. coli* isolates and is expressed in 31–44% of cystitis and 36–48% of pyelonephritis human clinical UPEC isolates (Mitsumori et al. 1999; Yamamoto et al.1995; Andreu et al. 1997; Michaud et al. 2017). CNF1 it belongs to a family of single chain AB-toxins, which enter mammalian cells by receptor-mediated endocytosis (Reppin et al. 2017; Sawaroska et al. 2019). CNF1 has been shown to activate Rho GTPases, contribute to urothelial cell invasion, induces stress fiber formation in endothelial cells to promote bacterial invasion and have cytotoxic effects on urothelium (Doye et al. 2002; Michaud et al. 2017; Saworoska et al. 2019). The toxicity of CNF1 is due to its ability to activate the Rho family GTPases, which promote activation of host cells membrane, and DNA replication in absence of cell division (Michaud et al. 2017). Rho family GTPases are molecular switches, which are tightly controlled by three groups of proteins: guanine nucleotide exchange factors (GEFs), which activate Rho proteins by GDP/GTP-exchange; GTPase-activating proteins (GAPs), which stimulate GTP hydrolysis and thereby control the inactivation of Rho GTPases; and guanine nucleotide dissociation inhibitors (GDIs), which predominantly bind the inactive form of Rho GTPases and block the nucleotide exchange (DerMardirossian et al. 2005; Reppin et al. 2017). Futhermore, CNF1 and HlyA are

normally detected in UPEC isolates and are co-expressed as closely linked genes (Falbo et al. 1992; Bigen-Bidois et al. 2002; Michaud et al. 2017). However, regarding hemolytic isolates from ExPEC infection, such UTI, a large proporcion of isolates encode also the *cnf1* gene, but is rarely detected in non-hemolytic UPEC isolates (Michaud et al. 2017). HlyA and CNF1 are also virulence factors frequently detected in *E. coli* isolates from companion animals with UTI (Féria et al. 2001; Siqueira et al. 2009; Mateus et al. 2013).

Regarding the cytolethal distending toxins (CDTs), these toxins were initially identified in *E. coli* from children with diarrhoea (Johnson and Lior 1987), however in the last years have been isolated also from humans with sepsis and urinary tract infection (Hinenoya et al. 2017). CDTs are encoded by *cdt* genes and have DNase activity leading to cellular and nuclear distension, resulting in irreversible cell cycle arrest and apoptosis of target cells (Meza-Segura et al. 2017). CDT of *E. coli* is divided into five types (CDT-I to CDT-V) based on differences in nucleotide sequences and its genomic location (Hinenoya et al. 2017; Meza-Segura et al. 2017).

CDTs are heterotrimeric holotoxins, consisting of CdtA, CdtB and CdtC subunits, and induce distention and death of certain cultured eukaryotic cell lines by arresting the cell cycle irreversibly at the G_1 or G_2 phase (Jinadasa et al. 2006; Yamasaki et al. 2006; Hinenoya et al. 2017). CdtB is the active subunit which can cause damage to the DNA through its DNase activity, while CdtA and CdtC subunits are responsible for binding to target cells and intracellular delivery of the CdtB subunit (Lara-Tejero and Galan 2001; Fedor et al. 2013; Hinenoya et al. 2017). CDT intoxication of epithelial cells leads to nuclear and cytoplasmic distension, formation of actin stress fibres and nuclear fragmentation, resulting in irreversible cell cycle arrest and death of target cells (Fedor et al. 2013; Grasso et al. 2015; Taieb et al. 2016; Meza-Segura et al. 2017). It has been considered that CDT is associated with colonization and invasion of the bacteria (Johnson and Stell 2000; Fox et al. 2004; Ge et al. 2007; McAuley et al. 2007).

The secreted enterotoxin TieB, encoded by the virulence gene *senB*, plays a role in the development of severe diarrhoea in patients infected by enteroinvasive *E. coli* (EIEC) strains (Nataro et al. 1995). The *senB* gene has been found on a large conjugative plasmid which may facilitate its dissemination among bacteria (Nataro et al. 1995) and located downstream to the *cjrABC* operon (for colicin Js receptor) (Smajs and Weinstock 2001). The *cjrABC* operon encodes for proteins that have been described to be involved in colicin Js uptake (Smajs and Weinstock 2001). Colicin Js is a polypeptide toxin and bind to bacterial receptors whose primary function is often to facilitate the uptake of nutrients, such as, vitamin B_{12} and may be be involved in iron acquisition (Smajs and Weinstock 2001), which may contribute to urovirulence (Mao et al. 2012). *SenB* gene has been found in UPEC isolates from humans and it has been associated with the phylogenetic group D (Cusumano et al. 2010; Mao et al. 2012). However, its role in UTIs is not clear until now.

Regarding the AT proteins, these proteins are a large family of proteins from the Order Enterobacterales that are translocated by a dedicated type V secretion system, also known as the autotransporter pathway (Dautin et al. 2010; Ruiz-Perez and Natarro 2014; Abreu et al. 2015; Abreu et al. 2016) and are normally associated with virulence functions (adhesion, aggregation, invasion, biofilm formation and toxicity) (Dautin et al.2010; Ruiz-Perez et al. 2014; Abreu et al. 2015; Abreu et al. 2016). AT translocation requires proteins, such the β-barrel assembly module and the translocation and assembly module (Ieva and Bernstein 2009; Sauri et al. 2009; Selkring et al. 2012). These proteins display the typical features of autotransporters: an N-terminal signal sequence, a passenger domain secreted into the extracellular medium and a C-terminal β-barrel domain involved in protein translocation through the outer membrane (Toloza et al. 2015). Furthermore, AT proteins consist of three main groups according to their structure, activity, and phylogenetic criteria: (1) a signal peptide that targets the protein to the secretory apparatus for inner membrane translocation; (2) a passenger domain that comprises the functional domain of the protein, and (3) a translocator domain that inserts into the outer membrane (Nichols et al. 2016). One major subgroup of AT proteins is the serine protease AT proteins of Enterobacterales (SPATEs) (Abreu et al 2015; Abreu et al. 2016; Nichols et al.2016). SPATEs are characterized by the presence of an immunoglobulin A1-like protease domain (PF02395) within the passenger domain that contains the conserved serine protease motif GDSGS (Yen et al. 2008; Dautin 2010; Nichols et al. 2016). Moreover, SPATEs can be phylogenetically grouped into two classes: class 1, which represent the major group of AT proteins and have cytotoxic activity (Henderson et al. 1999; Guyer et al. 2000; Dutta et al. 2002) and class 2, that comprises non-cytotoxic proteins with roles in colonization and immunomodulation (Toloza et al. 2015). This class recognize a more diverse range of substrates, such mucins (Yen et al. 2008; Ruiz-Peres and Natarro 2014; Nichols et al.2016) and immunomodulatory proteins (Ruiz-Perez et al. 2011; Nichols et al. 2016).

The serine protease autotransporters of the Enterobacterales represent a large family of virulence factors, which members resemble those belonging to the trypsin-like superfamily of serine proteases (Henderson et al. 2004; Abreu et al. 2015; Abreu et al. 2016). The serine protease autotransporters can be divided into two classes: classe 1-cytotoxins group and class 2- lectin-like immunomodulators group (Dautin 2010; Ruiz-Perez and Natarro 2014; Abreu et al. 2016). One important member of the class 2 is the protein involved in intestinal colonization (Pic), which was originally identified in cultures of EAEC isolates (Henderson et al. 1999; Abreu et al. 2015; Abreu et al. 2016), *Shigella flexneri* (Rajakumar et al. 1997; Abreu et al. 2016), UPEC isolates (Parham et al. 2004) and the hybrid EAEC/Shiga toxin-producing *E. coli* (serotype O104:H4) (Rasko et al. 2011; Munera et al. 2014; Abreu et al. 2016). Pic presents proteolytic activity on mucin and induces mucus hypersecretion (Henderson et al. 1999; Dutta et al. 2002; Gutiérrez-Jiménez et al. 2008; Navarro-Garcia et al. 2010; Abreu et al. 2015; Abreu et al. 2016) contributing to the mucosal colonization by enteroaggregative *E. coli* (EAEC) strains. Several

biological roles for Pic were described, including, serum resistance, hemagglutination, degradation of coagulation factor V, mucinolytic activity and cleavage of surface glycoproteins involved in leukocyte trafficking, migration, and inflammation (Henderson e al. 1999; Dutta et al. 2002; Parham et al. 2004; Gutiérrez-Jiménez et al. 2008; Ruiz-Perez et al. 2011; Abreu et al.205; Abreu et al. 2016; Sarowska et al. 2019). Furthermore, Pic-producing *Enterobacteriales* can disrupt the epithelial barrier, causing bacterial persistence, invasion, migration into the urinary tract, and the ability to cause bacteremia and sepsis (Abreu et al. 2015). Furthermore, Pic also promotes intestinal colonization of mice and rabbits, causing mucus hypersecretion and an increase in the number of mucus-producing goblet cells (Harrington et al. 2009; Navarro-Garcia et al. 2010; Munera et al. 2014).

The vacuolating autotransporter toxin (Vat) is a cytotoxin of class 2, which contributes to UPEC fitness during systemic infection (Nichols et al. 2016). This toxin has been shown to be most prevalent in ExPEC isolates from the B2 phylogenetic group of the ECOR collection (Parham et al. 2005). Furthermore, a high prevalence of this toxin has been observed in B2 isolates, with similar prevalences among cystitis, pyelonephritis, prostatitis, and bloodstream isolates (Parham et al. 2005; Nichols et al. 2016; Sarowska et al. 2019). Moreover, also has been associated with avian pathogenic *E. coli* (APEC) strains (Spurbeck et al. 2012; Zhao et al. 2015; Paixão et al. 2016).

In addition to the Pic and Vat proteins, other serine protease autotransporter is associated with urinary tract infection isolates of *E. coli,* the secreted autotransporter toxin (Sat). Sat is a vacuolating cytotoxin, causes cytopathic effects on various cell types and damage to kidney epithelium during upper urinary tract infection (Guyer et al. 2002). Sat protein is expressed significantly more often by *E. coli* isolates associated with the clinical symptoms of acute pyelonephritis than by fecal isolates from humans (Guyer et al. 2000). The native Sat protein (142 kDa) includes the three characteristic domains of SPATE proteins. The mature Sat protein (107 kDa) was shown to have a cytopathic effect on various cell lines (Guyer et al. 2000; Guyer et al. 2002) and to elicit glomerular damage and a vigorous antibody response in mice transurethrally infected with *E. coli* CFT073. Furthermore, the *sat* gene was detected in $PAI_{ICFT073}$ (Guyer et al. 2000, Maroncle et al. 2006).

Maroncle et al (2006) demonstrate that the serine protease active site of Sat is necessary for protease and cytotoxic activities, contraction of the cytoskeleton, and loss of actin in cultured human bladder and kidney cells but not for the processing or the release of the toxin from the bacterial surface (Maroncle et al. 2006). Futhermore, wild-type Sat is able to degrade specific membrane/cytoskeletal and nucleus-associated proteins (Maroncle et al. 2006). Moreover, the cytoskeletal effects mediated by Sat on urinary epithelial cells are associated with the degradation of fodrin (nonerythrocyte spectrin). Fodrin/spectrin is involved in stabilizing membrane structures, maintaining cell shape, and linking actin filaments with the plasma

membrane (Coleman et al., 1989, Beck and Nelson 1996; Maroncle et al. 2006). Alteration of the cytoskeleton may be the major mechanism for the host cell cytopathic effects caused by Sat toxin (Maroncle et al. 2006). Some *in vitro* studies demonstrate that Sat from UPEC strains displays proteolytic activity on casein, spectrin, fodrin and coagulation factor V, but Mucin, and IgA were not degraded (Guyer et al. 2000; Dutta et al.2002; Maroncle et al. 2006). Furthermore, in some cellular models of kidney, bladder and undifferentiated epithelial cells, Sat toxin promotes vacuolization, autophagy and cell detachment (Guyer et al. 2002; Liévin-Le Mola et al. 2011; Toloza et al. 2015).

1.8.5 Other virulence factors

Other important virulence factors that have been associated with ExPEC and/or UPEC are the arginine deiminase operon (*arcACBDR* operon); the invasion of brain endothelium; the Pathogenicity-asociated island marker of CFT073; the uropathogenic specific protein (usp) and the *E. coli* commom pilus (ecp) (Table 6).

The arginine deiminase operon (*arcACBDR* operon), which encodes proteins involved in an arginine deiminase pathway. This pathway comprises three reactions catalyzed by arginine deiminase, ornithine transcarbamoylase, and carbamate kinase (encoded by arcA, arcB, and arcC, respectively), resulting in the conversion of arginine into ornithine, NH3, CO2, and the production of ATP (Abdelal 1979; Billard-Pomares et al. 2019). The *arcACBDR* operon also contains genes encoding the ArgR and ArcD proteins, involved in the regulation of the operon and transport of arginine, respectively (Cunin et al. 1986).

Epidemiological studies showed that the *arcACBDR* operon is associated with the production of ESBLs, such CTX-M-producing *E. coli* isolates and increased over time (Billard-Pomares et al. 2019). This operon is highly mobile and can be found in a region framed by diverse ISs and carried by various genetic supports, such IncFII plasmids and various regions of the chromosome (Billard-Pomares et al. 2019). Furthermore, Billard-Pomares et al. (2019) demonstrate in a mouse UTI model, that an *E. coli* strain with the *arcACBDR* operon can use arginine as a carbon source. Moreover, this study shows complex gene-by-environment interactions in which the presence of the *arcACBDR* operon can be advantageous or not, depending on the organ and physiology (Billard-Pomares et al. 2019).

The *E. coli* common pilus (ecp), encoded by the *ecpRABCDE* operon, is an extracellular adhesive fiber first documented in association with *E. coli* isolates causing newborn meningitis and septicaemia (NMEC), where it was originally named the Mat (meningitis-associated and temperature-regulated) fimbriae (Pouttu et al. 2001). However, further studies have revealed that the ecp operon is ubiquitous across *E. coli*, making it the most common fimbrial structure in both commensal and pathogenic isolates (Pouttu et al. 2001; Rendón et al. 2007; Blackburn et al. 2009; Saldaña et al. 2009; Avelino et al. 2010;) and that ecp plays a dual role in early-stage

biofilm development and host cell recognition (Lehti et al. 2010). The majority of ecp is composed of a unique ~18-kDa protein called ecpA (Pouttu et al. 2001). This pilus playing a role in the adhesion of *E. coli* to the intestinal epithelium and the colon functions are act as a reservoir that can be responsible for the recurrent UTIs and allows some mechanisms to evade the immune system from their hosts (Rendón et al. 2007; Narciso et al. 2012). Furthermore, the *ecpA* gene is kept in intestine and can confer protection to antimicrobials, which can reduce the therapeutic options and the dissemination of ESBLs (Rendón et al. 2007; Narciso et al. 2012).

The uropathogenic specific protein (usp) and three OrfU proteins (OrfU1, OrfU2 and OrfU3) are encoded in the putative small pathogenicity island which is closely associated with UPEC (Parret and De Mot 2002; Zaw et al. 2013). Although homology analysis revealed that Usp and OrfUs have a homology with nuclease-type bacteriocins, which possess H-N-H nuclease motif, and immunity proteins respectively (Parret and De Mot 2002; Zaw et al. 2013). The H-N-H motif is known as a divalent metal ion binding, nucleic acid cleavage-module consisting of 30 to 40 amino acid residues. This motif could be observed in various types of nucleases represented by nuclease-type bacteriocins (Cheng et al. 2002; Walker et al. 2002) and intron-encoded homing endonucleases (Chevalier and Stoddard 2001). The C-terminal region of usp shows the homology to H-N-H motif (Parret and De Mot 2002). However, the H-N-H motif conserved in the C-terminal region of the Usp was indispensable for its nuclease activity, indicating usp is the new family member of H-N-H nuclease superfamily. Although, the *usp* gene was reported to be more frequently associated with UPEC strains than fecal *E. coli* isolates and enhance the infectious potential of *E. coli* strains in mouse pyelonephritis model, suggesting that may play a role in UPEC pathogenesis (Yamamoto et al. 2001). This gene is a genotoxin active against mammalian cells associated with isolates from pyelonephritis, prostatitis, bacteremia of urinary tract origin (Kurazono et al. 2000; Bauer et al. 2002; Ostblom et al. 2011; Črnigoj et al. 2014) and can induces a response characteristic of apoptosis (Nipič et al. 2013; Črnigoj et al. 2014). However, there is a possibility that usp also participates in infections outside of urinary tract because *usp* gene can be detected in some non-UPEC isolates (Zaw et al. 2013). However, the usp protein role and mechanisms of action, are not clear until now.

1.9 International high risk-clones of multidrug-resistant *Escherichia coli*

International multidrug-resistant (MDR) high-risk clones have a global distribution and can remain viable for prolonged time periods in diverse areas (Mathers et al. 2015b) and have been a leading cause of human community acquired infection and animal infections worldwide, including UTIs (Ewers et al. 2014; Marques et al. 2016; Marques et al. 2018).

Classification as an international multidrug-resistant high-risk clone, clones requires some criteria: (i) have a global distribution, (ii) an association with multiple antimicrobial resistance determinants, (iii) have the ability to colonize and persist in hosts for over 6 months, (iv) be

capable of effective transmission among hosts, (v) have enhanced pathogenicity and fitness and (vi) have the ability to cause severe and/or recurrent infections (Mathers et al. 2015b).

International clones of MDR *E. coli* strains belonging to sequence types (STs) *E. coli* ST38, ST69, ST131, ST155, ST393, ST405, and ST648. These clones have been emerging as a versatile prototype of MDR pathogens for human and animal hosts (Mathers et al. 2015b).

1.9.1 *Escherichia coli* clonal group, sequence type 131

MDR ESBL-producing *E. coli* ST131 is a high-risk ExPEC lineage that has spread explosively throughout the world (Rogers et al. 2011; Colpan et al. 2013; Banergee and Johnson 2014; Nicolas-Chanoine et al. 2014; Mathers et al. 2015; Pitout and Devinney 2017; Kondratyeva et al. 2020) mostly by patient-to-patient and food spread (Platell et al. 2011). This lineage colonizes healthy humans and animals (Nicolas-Chanoine et al. 2014) and is the causative pathogen of urinary tract and blood stream infections (Manges et al. 2009; Roger et al. 2011; Kudinha et al. 2013; Banergee and Johnson 2014; Nicolas-Chanoine et al. 2014; Mathers et al. 2015; Johnson et al. 2017; Kanamori et al. 2017 Pitout and Devinney 2017; Kondratyeva et al. 2020).

It was first identified in 2008 on different continents, such as in Norh America, Europe, Asia, Africa and Oceania (Coque et al. 2008; Lau et al. 2008; Nicolas-Chanoine et al. 2008; Nicolas-Chanoine et al. 2014), belongs to phylogroup B2 and are serotype O16:H5 or O25b:H4 (Nicolas-Chanoine et al. 2014) (Figure 7).

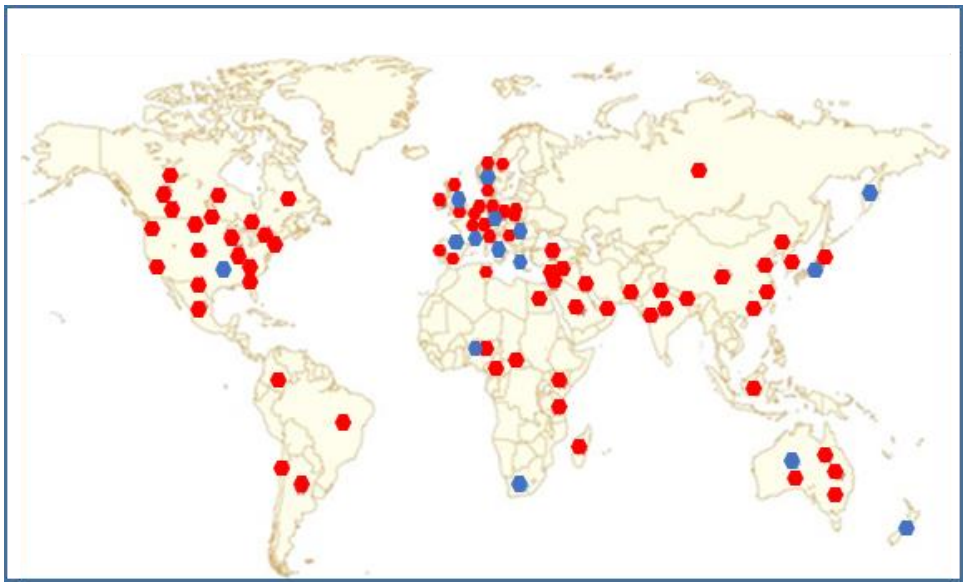


Figure 7: Global dissemination of *Escherichia coli* ST131 clone until 2013 (adapted from Nicolas-Chanoine et al. 2014).

Legend: Red color indicate isolates producing ESBL enzymes, and blue color indicate fluoroquinolone-resistant, non-ESBL-producing isolates.

E. *coli* ST131 is recognized as the major *E*. *coli* lineage responsible for the spread of MDR and in specific CTX-M ESBL genes (Mathers et al. 2015b; Doi et al. 2017; Manges et al. 2019; Kondratyeva et al. 2020). The global expansion of this lineage is driven by spread of two major fluoroquinolone-resistance (FQ-resistant) clades which possess *fimH*30 allele - *H*30R/C1 (clade C1) and *H*30Rx/C2 (clade C2), where the later harbours $bla_{CTX\text{-}M\text{-}15}$ ESBL gene (Price et al. 2013; Nicolas-Chanoine et al. 2014; Johnson et al 2016b; Stoesser et al. 2016; Pitout and DeVinney et al. 2017). The major CTX-M genes described in clade C1 were CTX-M-14 and −27 alleles of the CTX-M-9 group (Matsumura et al. 2016), with this later allele- C1-M27 (Matsumura et al. 2016), recognized as a new subclade with global occurrence (Ghosh et al. 2017; Merino et al. 2018). Moreover, there are other two ST131 subclones less frequently expanded: *H*22 (clonotype CH40-22, clade B) and *H*41 (clonotype CH40-41, clade A). Isolates of subclone *H*41 usually belong to serotype O16:H5 (Price et al. 2013; Olesen et al. 2014; Peirano et al. 2014; Matsumura et al. 2017). Furthermore, plasmid associations in ST131 clades connected CTX-M-15-encoding IncF [F2:A1:B-] (FAB formula) plasmids to clade C2 (Price et al. 2013; Johnson et al. 2016; Stoesser et al. 2016; San Millan et al. 2018; Kondratyeva et al. 2020) and non-CTX-M-15-encoding IncF [F1:A2:B20] plasmids to clade C1 (Johnson et al. 2016b; Pitout and DeVinney 2017). Generally, clades A and B are fluoroquinolone (FQ) susceptibility and rarely carry ESBL plasmids, while most isolates of clade C are FQ-resistant. Clade B evolved into clade C by acquisition of several prophages, genomic islands, the *fimH*30 allele and mutations within *gyrA* and *parC* genes, mainly during the late 1980s (Stoesser et al. 2016; Pitout and DeVinney 2017; Kondratyeva et al. 2020). The population structure of the *E. coli* ST131 *fimH*30 lineage, *H*30 sublineages, and other ST131-associated lineages is illustrated in Figure 8.

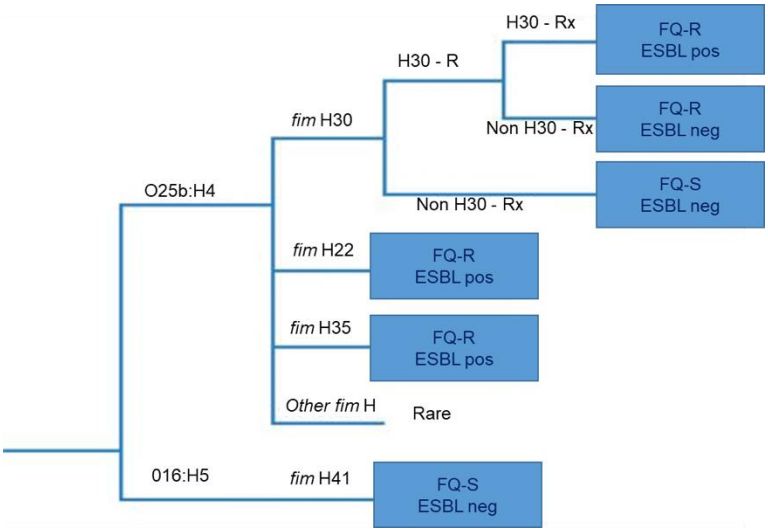


Figure 8: Population structure of the *Escherichia coli* ST131 fim*H*30 lineage, *H*30 sublineages, and other ST131-associated lineages (adapted from Mathers et al. 2015a).

Legend: FQ-R, fluoroquinolone resistant; FQ-S, fluoroquinolone susceptible.

In addition, considering the content of virulence genes, ST131 isolates can be classified into different virotypes (Table 7) (Blanco 2013; Nicolas-Chanoine et al. 2014).

Table 7: Virulence-gene scheme for defining ST131 *Escherichia coli* virotypes (adapted from Nicolas-Chanoine et al. 2014).

Virotype	Virulence factor-encoding gene									
	afa/draBC	*afa* operon	*iroN*	*sat*	*ibeA*	*papGII*	*cnf1*	*hlyA*	*papGIII*	*cdtB*
A	+	+	−	+/−	−	−	−	−	−	−
B	−	−	+	+/−	−	−	−	−	−	−
C	−	−	−	+	−	−	−	−	−	−
D	+/−	+/−	+/−	+/−	+	−	+/−	+/−	+/−	+/−
E	−	−	−	+	−	+	+	+	−	−

Legend: +, positive PCR result; −, negative PCR result. *afa/draBC*, Afa/Dr adhesins; *afa* operon,; *iroN*, catecholate siderophore receptor; *sat*, secreted autotransporter toxin; *ibeA*, invasion of brain endothelium; *papG*II, allele II of *papG* gene; *cnf1*, cytotoxic necrotizing factor type 1; *hlyA*, alpha-hemolysin; *papG*III, allele III of *papG* gene; *cdtB*, cytolethal distending toxin; *neuC*-K1, K1 variant of group II capsule.

ST131 isolates have also been detected in nonhuman sources such as companion animals, wildlife, avian, primate, food sources, and in environment sources (Platell et al. 2011; Vignaroli et al. 2013; Pomba et al. 2014b; Jamborova et al. 2018; Finn et al. 2019). The first report of an animal source of *E. coli* ST131 was published by Pomba et al. (2009) and concerned a dog suffering from a UTI in Portugal. The isolate obtained from the dog was resistant to FQ and harbored the $bla_{\text{CTX-M-15}}$, *qnrB2*, and *aac* (*6'*)-*Ib-cr* genes on an IncFII plasmid (Pomba et al. 2009). This finding was interpreted as indicating the possible entry of the emerging, virulent human ST131 clone into the animal population, due to the proximity between companion animals and their owners, facilitating human-to-animal transfer and vice-versa (Nicolas-Chanoine et al. 2014). Furthermore, until now there have been very few reports on *E. coli* ST131 from companion animals (Table 8).

Table 8: *Escherichia coli* ST131 in companion animals (adapted from Nicolas-Chanoine et al. 2014).

Country	Study period	Study design	ST131 isolates/ total	FQ^R	ESBL/AmpC genes in ST131	Sublineage	Reference
Portugal	2004-2006	61 UTI *E. coli* isolates among 41 clinical dogs and 20 cats.	1/61	Yes	CTX-M-15	NA	Pomba et al., 2009; Pomba et al. 2014,
United States	NA	Dogs and cats in the same household (index case plus colonization)	3/6	Yes	No	NA	Johnson and Clabots 2006
France	2006-2010	19 ESBL-producing *E. coli* isolates among 518 clinical dogs and cats	1/19	Yes	CTX-M-14	NA	Dahmen et al. 2013
Europe (8 countries, mainly Germany)	2008-2009	177 ESBL-producing *E. coli* isolates (mostly cats, dogs, horses)	10/177	Mostly yes	Mostly CTX-M-15	NA	Ewers et al. 2010
Japan	2011	33 cefazolin resistant ExPEC isolates, dogs and cats	4/33	ND	CTX-M-27	NA	Harada et al. 2012
Switzerland	2010-2011	107 UTI *E. coli* isolates, 59 dogs and 40 cats, 4 with ESBL/AmpC-producing *E. coli* isolates	0/107	NA	NA	NA	Huber et al. 2013
Korea	2006-2007	628 *E. coli*, 422 stray and 206 hospitalized dogs, 34 carrying ESBL/AmpC-producing *E. coli* isolates.	0/34	NA	NA	NA	Tamang et al. 2012
Netherlands	2007-2009	2700 clinical Enterobacteriales isolates from mostly dogs, cats, and horses, 65 carrying ESBL/AmpC-	1/65	Yes	CTX-M-15	NA	Dierikx et al. 2012

		producing E. coli isolates					
Kenya	2009	49 ESBL-producing *E. coli* isolates, dog and cat carriers	3/49	Yes	CTX-M-15	NA	Albrechtova et al. 2012
Japan	2015	178 *E. coli* isolates from dogs and cats from urine, pus and others	15/178	Yes	CTX-M-15 CTX-M-27	H30R H30Rx H30	Kawamura et al. 2017

Legend: UTI, urinary tract infection; ESBL, extended-spectrum β-lactamase; FQr, fluoroquinolone resistant; AmpC, cephalosporinase; ExPEC., extraintestinal pathogenic E. coli; NA, not available; ND, not determined.

1.9.2 *Escherichia coli* clonal group, sequence type 648 (ST648)

E. coli isolates belonged to the international high-risk clone sequence type ST648, has been described in human infections harbouring several β-lactamases, which has contributed to the spread of CTX-M-, CMY-2-, NDM-, OXA-48-, and MCR-1-type encoding genes (Poirel et al., 2018; Marques et al. 2018). Furthermore, fluoroquinolone resistant strains of ST648 also acquired resistance to the tetracycline derivative tigecycline via expression of efflux pump AcrAB-TolC (Sato et al. 2016).

This *E. coli* lineage has emerged as a pandemic clone, being globally reported in humans and companion animals (healthy and diseases) in Asian, European, American and Oceania countries (Tamang et al. 2012; Huber et al. 2013; Ewers et al. 2014; Toleman et al. 2015; Gonçalves et al. 2016; Li et al. 2017; Liu et al. 2016; Solgi et al. 2017; Marques et al. 2018; Sellera et al. 2018; Fernandes et al. 2018b). The distribution of ESBL/AmpC-producing ST648 strains among companion animals, are an important source of human infections and vice-versa. Yet, have been very few reports on *E. coli* ST648 from companion animals (Table 9).

Table 9: *Escherichia coli* ST648 in companion animals.

Country	Study period	Study design	ST648 isolates /total	ESBL/AmpC genes in ST648	Reference
South Korea	2006-2007	628 *E. coli* isolates recovered from intestinal samples from dogs. 23 AmpC β-lactamase-producing E. coli isolates	9/23	CMY-2	Tamang et al. 2012
Europe (11 countries, mainly Germany)	2008-2011	1152 ESBL-producing *E. coli* isolates from dogs companion animals and horses.	30/1152	CTX-M-15, CTX-M61, CTX-M-14, CTX-M-1	Ewers et al. 2014
United States	2009-2013	2443 *E. coli* isolated from urine, wound, ear, genital tract, nasal structure, and soft tissue samples of dogs and cats. 68 ESBL-producing *E. coli.*	9/68	CMY-2, SHV-12, CTX-M-9, CTX-M-1+CTX-M-15, CTX-M-9+CTX-M-14, OXA-48	Liu et al. 2016
China	2011-2013	118 UTI *E. coli* isolates, from dogs. 3 carrying ESBL -producing *E. coli* isolates	1/3	CTX-M-15	Li et al. 2017
Portugal	1999-2014	412 UTI *E. coli* isolates, from dogs and cats. 27 ESBL/AmpC -producing *E. coli* isolates	10/27	CMY-2 and CMY+CTX-M-9	Marques et al. 2018
South America	2017	20 free-roaming cats	2/20	CTX-M-2 and CTX-M-15	Fernandes et al. 2018b

1.9.3 *Escherichia coli* clonal group, sequence type 410 (ST410)

Some studies indicate that *E. coli* ST410 is another successful pandemic extraintestinal pathogenic *E. coli* (ExPEC) lineage similar to ST131 (Falgenhauer et al. 2016a; Schaufler et al. 2016). *E. coli* ST410 was first identified in 2016 in China (Qin et al. 2016) and since them and has been reported worldwide (Roer et al. 2018). Accordingly with Roer (2018) there are two main clades, clade A (*fimH*53) and clade B (*fimH*24), with B clades subdivided based on different antimicrobial resistance characteristics: the B2/*H*24R sublineage with acquired fluoroquinolone resistance by mutations in *gyr*A and *par*C and the B3/*H*24Rx sublineage with acquired $bla_{\text{CTX-M-15}}$ gene from the IncFII plasmid harboring FIA/FIB replicons; B4/*H*24RxC sublineage with acquired $bla_{\text{OXA-181}}$ gene from the IncX3 plasmid and acquired $bla_{\text{NDM-5}}$ gene from the IncF replicon (Roer et al. 2018). Nowadays, two sublineages are currently circulating in Europe and North America, the B3/*H*24Rx the B4/*H*24RxC) (Roer et al. 2018; Patiño-Navarrete et al. 2020). Furthermore, some ST410 strains from Germany and Brazil have been reported to have the mobile colistin resistance gene *mcr*-1 (Falgenhaueur at al. 2016b; Rocha et al. 2017).

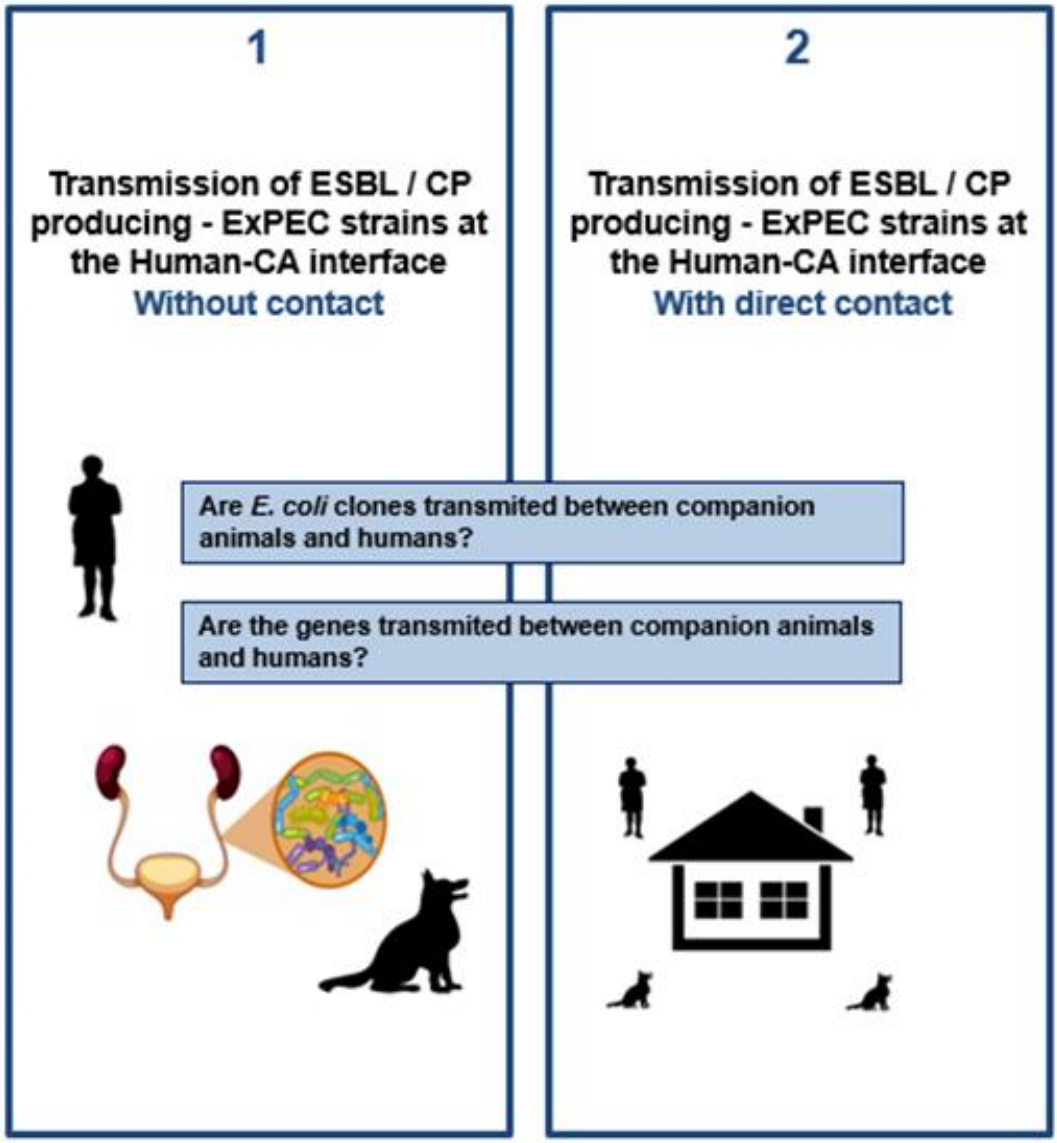
E. coli ST410 strains are transmissible between humans, companion animals, wildlife and the environment (Schaufer et al. 2016; Falgenhaueur at al. 2016b; Nigg et al. 2019) and is already globally distributed, reported in different countries. However, regarding companion animals few reports about this clone have been published.

Chapter 2

Study goals

The major goal of this thesis was to assess the prevalence and to determine characteristics of ESBLs/ carbapenemase-producing Extraintestinal pathogenic *Escherichia coli* (ExPEC) in companion animals and humans without direct contact and in direct contact and its public health relevance. The experimental work was divided in two main parts:

- In the first part of this thesis, the aim was to characterize ESBLs/ carbapenemase *E. coli* strains from companion animals and humans with UTI and the ESBLs/ carbapenemase-producing *E. coli* faecal colonization in healthy companion animals. By studying the antimicrobial resistance, virulence genotype and population structure of strains of companion animals and humans without living in direct contact. This part of the study aimed to get insights on the role of companion animals as reservoirs and in the dissemination of clinical strains of *E. coli*.
- The second part of this work aimed to characterize the gut colonization by *E. coli* and the resistance genes transmitted between companion animals and humans living in close contact. This part of the thesis aimed at providing new data about clinically important antimicrobial resistance genes and ESBL/Carbapenemase-producing-pathogenic *E. coli* from companion animal-human sharing and assess the role of companion animals as reservoirs to humans and vice-versa.


1
Transmission of ESBL / CP producing - ExPEC strains at the Human-CA interface
Without contact
2
Transmission of ESBL / CP producing - ExPEC strains at the Human-CA interface
With direct contact
Are E. coli clones transmited between companion animals and humans?
Are the genes transmited between companion animals and humans?

Chapter 3

ESBLs/ carbapenemase - producing Extraintestinal pathogenic *Escherichia coli* in companion animals and humans without direct contact

3.1. ESBLs/ carbapenemase *Escherichia coli* strains from companion animals and humans with UTI.

3.1.1- Emergence of *Escherichia coli* ST131 H30/H30-Rx subclones in companion animals.

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Partial results were presented as,

Two Oral communications at the International congress at the 1st International Conference of the European College of Veterinary Microbiology, 2019, Athens Greece and at 27th European Congress of Veterinary Internal Medicine – Companion Animals (ECVIM-CA), 2017, St. Julians, Malta.

Three Poster communications at the International congress International Society of Feline Medicine World Feline Congress 2017, 2017, Brighton, United Kingdom; International congress 26th ECCMID, 2016, Amsterdam, the Netherlands; and at the International congress ASM Microbe 2016, 2016, Boston USA.

Emergence of *Escherichia coli* ST131 H30/H30-Rx subclones in companion animals.

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The *Escherichia coli* O25b-ST131, with its fluoroquinolone-resistant *H*30 subclone and its nested ESBL CTX-M-15-associated *H*30-Rx subclone, is the most disseminated MDR and virulent *E. coli* clonal group worldwide.[1] In previous work, we have reported the first detection of this O25b-ST131 clone causing urinary tract infection (UTI) in a dog.[2] Additionally, later on, we found within-lineage variability of ST131 *E. coli* UTI isolates from humans and companion animals by PFGE analysis.[3] The detection of human high-risk pandemic *E. coli* clones causing UTI in companion animals is a great public health concern.[4]

Between 1999 and 2015, 342 uropathogenic *E. coli* were isolated at the Laboratory of Antimicrobial Resistance, Faculty of Veterinary Medicine, University of Lisbon from companion animals (dogs and cats) with UTI. Significant bacteriuria was determined based on a quantitative urine culture according to the urine collection method used (puncture, catheter or free catch). Urine samples were collected from companion animals at the teaching hospital of the Faculty of Veterinary Medicine and at private veterinary hospitals in Lisbon, Portugal.

All B2 phylogroup isolates were studied by PCR for the ST131-associated SNP in the *mdh* and *gyrB* genes; [5] 14.5% ($n = 25/172$) were the pandemic O25b:H4-B2-ST131 clone. The *E. coli* O25b:H4-B2-ST131 *H*30 and *H*30-Rx subclones were screened by PCR as previously described.[6] Seven ($n = 7/25$; 28%) of the O25b:H4-B2-ST131 clone companion animal isolates were the *H*30 subclone and three out of these seven were the *H*30-Rx subclone (Table 1). To the best of our knowledge this is the first report of *E. coli* ST131 *H*30/*H*30-Rx subclones causing UTI in companion animals in Europe.

The presence of uropathogenic *E. coli* (UPEC) virotype markers was assessed, i.e. Pap fimbriae (*papEF* operon segment), Sfa fimbriae and Afa afimbrial adhesin (*sfa* and *afa* genes, respectively), the *hlyA* gene from the α-haemolysin operon, cytotoxic necrotizing factor 1 (*cnf1* gene), aerobactin siderophore (*iucD* gene), *E. coli* common pilus (*ecpA* gene) and the uropathogenic specific protein (*usp* gene).[7,8] The distribution of alleles I, II and III of the P adhesin gene *papG* was studied.[9]

Additionally, pathogenicity-associated islands (PAIs) (PAI_{IV536}, PAI_{I536}, PAI_{II536}, PAI_{III536}, PAI_{IJ96}, PAI_{IIJ96}, $PAI_{ICFT073}$, $PAI_{IICFT073}$) were identified.[10] The *E. coli* O25b:H4-B2-ST131-*H*30/*H*30-Rx most common pathogenicity and virulence-associated gene profiles were $PAI_{ICFT073}$-PAI_{IV536}-

$PAI_{IICFT073}$ (42.9%, *n* = 3/7) and *ecpA-hlyA-papEF-iucD* (28.6%, *n* = 2/7), respectively. The full pathogenicity and virulence genotype of *E. coli* O25b:H4-B2-ST131-*H*30/*H*30-Rx is shown in Table 1. All *H*30/*H*30-Rx subclones causing UTI in companion animals belonged to virotype D, which confirms their virulent characteristics.[1]

Resistance to third-generation cephalosporins was detected only in the three isolates of the *H*30-Rx subclone. β-Lactamase genes were screened as reported elsewhere.[2] As expected from other studies, O25b:H4-B2-ST131 *H*30 and *H*30-Rx isolates were fluoroquinolone resistant,[1] yet one O25b:H4-B2-ST131-*H*30-Rx *E. coli* did not carry the ESBL $bla_{CTX\text{-}M\text{-}15}$, but instead carried the frequent $bla_{CTX\text{-}M\text{-}1}$ gene, which is associated with *E. coli* isolates of farm animal origin and recently described in humans in Turkey with UTI.[1,11] The mechanism of resistance to other antimicrobial classes was characterized by PCR and nucleotide sequencing for *sul1*, *sul2*, *sul3*, *dfrA1*, *dfraA12* and the plasmid-mediated quinolone resistance (PMQR) genes [*qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD*, *qepA*, *aac(6')-Ib* and the MDR *oqxAB* genes of the efflux pump].[12] The *aac(6')-Ib-cr* gene was the most common PMQR gene detected (57.1%, *n* = 4/7) and was more frequently found in ST131 *H*30-Rx *E. coli* isolates than in ST131 *H*30 isolates. The *qnrB2* and *aac(6')-Ib-cr* genes were detected in the FMV5825/04 dog isolate as expected from previous data (Table 1).[2]

In Europe, carbadox and olaquindox, which are quinoxaline derivatives with antibacterial properties, were used for the prevention of dysentery and as growth promotors in pigs since 1974 and 1976, respectively. Fortunately, their use has been banned in farm animals in Europe for decades, but not in China.[13] The efflux pump OqxAB also extrudes antibiotics such as chloramphenicol and fluoroquinolones. Isolate FMV5695/09 O25b:H4-B2-ST131-*H*30 harboured both *oqxA* and *oqxB* efflux pump genes, which could potentially be related to the reduced fluoroquinolone susceptibility. To the best of our knowledge, this is the first description of an ST131 *E. coli* harbouring the *oqxAB* efflux pump. Further studies will be necessary to elucidate the importance of this resistance mechanism in the ST131 pandemic *E. coli* clone.

This study reports the detection and frequency of the *E. coli* O25b:H4-B2-ST131 *H*30/*H*30-Rx subclones in companion animals with UTI in Portugal and, to the best of our knowledge, in Europe. In conclusion, the findings presented in this study are relevant and, to the best of our knowledge, represent the first detection of ST131 *H*30/*H*30-Rx *E. coli* isolates associated with UTI in companion animals in Europe. To the best of our knowledge, this is the first report in Europe of the disseminated *E. coli* O25b:H4-B2-ST131-*H*30/*H*30-Rx, MDR, fluoroquinolone-resistant human high-risk clone and its CTX-M-15-*H*30-Rx and CTX-M-1-*H*30-Rx subsets in companion animals with UTI. Studies of ST131 *H*30/*H*30-Rx in humans in Portugal are scarce, yet *H*30/*H*30-Rx subclones have been described in faecal samples in healthy humans.[14] These results raise public health concerns since these subclones may have an impact on human health through the close and direct contact between companion animals and owners.

Moreover, the close contact between companion animals and humans creates opportunities for interspecies transmission of resistant bacteria.

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Transparency declarations

Nothing to declare.

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Table 1. Characterization of *Escherichia coli* O25b:H4-B2-ST131-*H*30 and *H*30-Rx subclones isolated from companion animals.

Isolate	Year	Companion Animal origin	Clonal group ST131	Subclone *H*30	Subclone *H*30Rx	Antimicrobial resistance[a]	ESBL and/or *p*AmpC genes	Other resistance genes	PAIs	Virotype[b]	Virulence genes
FMV2358/03	2003	Dog	YES	YES	NO	AMP-TE-CIP-ENR	NO	-	PAI_{II536}- $PAI_{II\ J96}$- PAI_{IV536}- $PAI_{IICFT073}$	D	*ecpA-hly*A-*cnf*1-*sfa*DE-*pap*EF-*papG* III-*iuc*D
FMV5825/04	2004	Dog	YES	YES	YES	AMP-AMC-KF-CTX-CAZ-FOX-SXT-TE- CIP-ENR-CN-AK-TOB	$bla_{CTX\text{-}M\text{-}15}$	*bla*$_{TEM}$ *bla*$_{OXA\text{-}1}$-*sul1*-*aac(6´)-Ib-cr*-*qnrB2*	PAI_{II536}- $PAI_{II\ J96}$- $PAI_{ICFT073}$-PAI_{IV536}- $PAI_{IICFT073}$	D	*ecpA-hly*A-*cnf*1-*sfa*DE-*pap*EF-*iuc*D
FMV2777/08	2008	Cat	YES	YES	YES	AMP-AMC-KF-CTX-CAZ-TE-CIP-ENR-CN-TOB	$bla_{CTX\text{-}M\text{-}15}$	*aac(6´)-Ib-cr*	$PAI_{ICFT073}$-PAI_{IV536}-$PAI_{IICFT073}$	D	*ecpA-hly*A-*pap*EF- -*iuc*D
FMV6710/08	2008	Dog	YES	YES	NO	AMP-SXT-CIP-ENR	NO	*sul2*	$PAI_{ICFT073}$-PAI_{IV536}-$PAI_{IICFT073}$	D	*ecpA-hly*A -*pap*EF-*iuc*D
FMV5695/09	2009	Dog	YES	YES	NO	AMP-TE-CIP-ENR	NO	*bla*$_{TEM}$-*oqxAB*	PAI_{IV536}-$PAI_{IICFT073}$	D	*ecpA-pap*EF-*iuc*D
FMV58/13	2013	Cat	YES	YES	YES	AMP-KF-CTX-SXT-CIP-ENR	$bla_{CTX\text{-}M\text{-}1}$	*bla*$_{TEM}$ - *dfrAI*-*aac(6´)-Ib-cr*	$PAI_{ICFT073}$-PAI_{IV536}-$PAI_{IICFT073}$	D	*ecpA-hly*A-*sfa*DE-*pap*EF-*iuc*D
FMV146/14	2014	Dog	YES	YES	NO	AMP-TE- CIP-ENR	NO	*aac(6´)-Ib-cr*	PAI_{II536}-$PAI_{ICFT073}$-PAI_{IV536}	D	*ecpA-hly*A-*pap*EF-*afa*BC-*iuc*D

AMC- Amoxicilin/clavulanate, AMP-Ampicillin, AK- Amikacin, CAZ-Ceftazidime, CIP-ciprofloxacin, CN- gentamycin, CTX-Cefotaxime, ENR-Enrofloxacin, FOX-Cefoxitin, SXT-trimethoprim/sulphamethoxazole, TE- tetracycline, TOB-Tobramycin; [a]susceptibility was accessed according to Clinical and Laboratory Standards Institute (CLSI) guidelines M100-S27. [b]Classification of the virotype according to Nicolas-Chanoine et al.[1]

3.1.2 - Public health impact of ESBLs/pAmpC- producing *Escherichia coli* causing urinary tract infections in non-related companion animals and humans.

Paper to be submitted at *Frontiers in Microbiology*.

Belas A, Menezes J, da Gama L.T, Carriço JA, Pomba C. Public health impact of ESBLs/pAmpC-producing *Escherichia coli* causing urinary tract infections in non-related companion animals and humans. Frontiers in Microbiology (paper to be submitted)

Partial results were presented as,

Two Oral communications at the International congress at the 1st International Conference of the European College of Veterinary Microbiology, 2019, Athens Greece and at 27th European Congress of Veterinary Internal Medicine – Companion Animals (ECVIM-CA), 2017, St. Julians, Malta.

Five Poster communications at the International congress 30th ECCMID, 2020, Paris, France; International congresso Microbiotec 2019; International Society of Feline Medicine World Feline Congress 2017, 2017, Brighton, United Kingdom; International congress 26th ECCMID, 2016, Amsterdam, the Netherlands; and at the International congress ASM Microbe 2016, 2016, Boston USA.

Two Poster communications at the National congress XII Congresso Hospital Veterinário Montenegro, Santa - Maria da Feira, Portugal.

Public health impact of ESBLs/pAmpC- producing *Escherichia coli* causing urinary tract infections in non-related companion animals and humans.

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Abstract

Objectives: This study aimed to characterize and compare ESBL/AmpC ß-lactamases producing - *E. coli* strains causing urinary tract infections (UTI) in companion animals (CA) and non-related humans from the community with UTI (H-CA).

Methods: Third generation cephalosporins (3GC) - resistant *E. coli* (companion animals *n*=35; humans *n*=85) isolated from patients with UTI were tested against 14 antimicrobials. PCR-based assays were used to detect the major *E. coli* phylogenetic groups, Pathogenicity associated-islands (PAIs), virulence genes and ESBLs/pAmpC resistance genes. ESBL/pAmpC-producing *E. coli* isolates were typed by multi locus sequence type (MLST). The ST131 clonal group and subclades C2 (*H*30-Rx) and C1 (*H*30-R1) were identified by PCR. Genetic relationships among *E. coli* isolates were visualized by the goeBURST algorithm based on the PHYLOViZ software (v.2). Molecular epidemiology was investigated using repetitive element sequence-based PCR (rep-PCR) typing method.

Results: The frequency of resistance against fluoroquinolones (CA=74.3%, H-CA=88.2%), trimethoprim/sulphamethoxazole (CA=71.4%, H-CA=74.1%) and gentamicin (CA=40%, H-CA=37.6%) were higher in 3CG-resistant *E. coli* from both groups. All isolates were susceptible to carbapenems. Considering phylogenetic group 3GC-resistant *E. coli* strains from companion animals and humans mainly belonged to group-D and B2 (48.6%, 67.1%, respectively). The most frequent PAIs and virulence genes from CA and H-CA were: PAI IV_{536} PAI I_{CFT073} (*p*=0.017, *p*=0.013 respectively), *ecp*A and *iuc*D (*p*=0.0002).

MLST typing of the ESBL/pAmpC producing *E. coli* strains revealed a heterogeneous population of *E. coli* clonal groups in companion animals and in humans with UTI. Companion animals and humans *E. coli* strains shared two MDR high-risk clonal lineages: ST131, and ST648, an emergent virulent lineage. The bla_{CMY-2} and $bla_{CTX-M-15}$ were the most frequently ESBL/pAmpC detected genes in companion animals and human strains. ST131 strains from companion animals and humans mostly belonged to subclade C2 (*H*30-Rx). rep-PCR analysis confirmed that ESBL/AmpC –producing *E. coli* strains from companion animals and humans with UTI that have the same MLST and combination of PAIs had identical fingerprints.

Conclusion: Considering that companion animals with UTI are generally treated at home by the owners, measures should be implemented to avoid the spread of multidrug-resistant high-risk clones to the house-hold environment.

Keywords: *Escherichia coli*, ESBL/AmpC, Pathogenicity, Clones, Companion animals, Humans, Urinary tract infection.

Introduction

Urinary tract infections (UTIs) are mostly caused by the uropathogenic *Escherichia coli* (UPEC), one of the extraintestinal pathogenic *E. coli* pathotypes (ExPEC) (Kaper et al., 2004). UTI is responsible for significant morbility and mortality and is one of the most common etiologic agents of UTI worldwide in humans and in companion animals (Foxman, 2010; Jakobsen et al., 2010; Marques et al., 2017).

The increase of antimicrobial resistance caused by multidrug-resistant (MDR) strains, such extended-spectrum beta-lactamases (ESBLs), cephalosporinases (AmpC), including cephalosporins, and carbapenems are increasing worldwide and also of problem to public health, because of their ability to cause treatment failure due to third- and fourth-generation cephalosporins, which have been considered highest priority critically important antimicrobials to human and veterinary medicine (WHO, 2018). Moreover, in the last years, some studies have alerted on the emergence of MDR high-risk clonal lineages of clinically significant bacteria in companion animals, raising public health concerns, since infected and colonized companion animals may contribute to the spread of such bacteria among humans, domestic animals, wildlife, environment and in food-chain production (Ewers et al., 2009; Ewers et al., 2010, Narciso et al., 2012; Marques et al., 2017). Important causes of high-risk clones dissemination is the production of β-lactamase (ESBLs/AmpC β-lactamases or cephalosporinases and carbapenemases) enzymes. The most prevalent of these are CTX-M-like enzymes as well as other types, such as: TEM and SHV enzymes and the plasmid-mediated AmpC. Moreover, association with various antimicrobial resistance determinants, pathogenicity, global distribution, the ability to colonize and persist in hosts for more of 6 months, capacity of transmission among hosts and the ability to cause recurrent infections, also are important factors to qualify as an international multidrug-resistant high-risk clone (Mathers et al., 2015).

The population structure of ESBL/pAmpC-producing *E. coli* is dominated globally by high-risk clones namely ST131, ST648, ST69, ST393, ST405, ST410 and other important clones. *E. coli* ST131 has been grouped into different clades, which are usually associated with specific fim*H* alleles: clade A (fim*H*41 ST131-O16), clade B (fim*H*22 ST131-O25b) and clade C (fim*H*30 including ST131-O25b fim*H*30-R/fim*H*30-Rx). Clade C1-M27 is associated with $bla_{\text{CTX-M-27}}$, and C2 with $bla_{\text{CTX-M-15.}}$ (Mathers et al., 2015; Peirano and Pitout, 2019).

With the growing contact between companion animals and humans, the risk of animal-to-human transfer of such bacteria is of concern (Pomba et al., 2017). Additionally, previous studies have demonstrated that companion animals may share UPEC with the remaining household members, indicating zoonotic transmission and humans may be a reservoir of UPEC for their companion animals as seen with *E. coli* ST131. (Johnson et al., 2009a; Johnson et al., 2009b; Pomba et al, 2014; Barroso et al., 2018; Belas et al., 2019).

Furthermore, there seems to be a match in the geographic distribution of the human and animal CTX-M enzymes, with CTX-M-1 predominating in Africa and Europe, CTX-M-14 in Asia and North America and CTX-M-15 β-lactamase in North America, Europe, and Africa (Bortolami et al., 2019). UPEC isolates usually have the largest number of pathogenicity-associated islands (PAIs) encoding a variety of virulence determinants encoding adhesins, toxins, invasins, other proteins and iron uptake systems that enable them to invade, colonize, and survive in the urinary tract, and prevent them from removal during urination from the host and consequently influencing the pathogenicity of symptomatic or complicated UTIs (Kaper et al., 2004). PAIs have been spread among bacteria by horizontal transfer and provide a virulence advantage allowing their adaptation to niches incapable to be colonized by commensal *E. coli* strains (Kaper et al., 2004; Sabaté et al., 2006; Lloyd et al. 2009).

With the growing contact between companion animals and humans, the risk of animal-to-human transfer of such bacteria is of concern (Pomba et al., 2017). Additionally, previous studies have demonstrated that companion animals may share UPEC with the remaining household members, indicating zoonotic transmission and humans may be a reservoir of UPEC for their companion animals as seen with *E. coli* O25b:H4-B2-ST131, this clonal group has dramatically spread during the last decades, also some lineages of this clone, such as *H*30Rx clade C2 (C2/*H*30Rx), have been linked to the dissemination of the extended-spectrum β-lactamases (ESBL), especially CTX-M-15 (Johnson et al., 2009a; Pomba et al, 2014; Barroso et al., 2018; Belas et al., 2019). However, research has been predominantly focused on ST131 and fewer studies in companion animals and humans have been done for the emerging high-risk clones ST648 and ST410 (Zong and Yu., 2010; Peirano et al., 2012; Ewers et al., 2014; Mathers et al., 2015; Marques et al., 2017; Schaufler et al., 2019).

Therefore, the characterization of *E. coli* isolated from companion animals with UTI, especially those harbouring important antimicrobial resistance mechanisms, is crucial to evaluate the extent to which companion animals with UTI may act as reservoirs for UPEC or vice- versa.

Antimicrobial resistance and ESBL/AmpC-producing *E. coli* is a complex subject and their emergence, ecology, and the association between virulence is poorly understood.

Thus, this study aimed to characterize and compare: ESBL/AmpC ß-lactamases producing *E. coli* strains causing urinary tract infections in companion animals and non-related humans in the community; Pathogenicity background, virulence profile and clonal groups determination of these strains.

Materials and Methods

Bacterial isolates

From 1999 to 2015, 330 non-duplicate uropathogenic *E. coli* (UPEC) were isolated at the Laboratory of Antibiotic Resistance from Faculty of Veterinary Medicine, University of Lisbon from

companion animals (dogs and cats) with UTI. The significant bacteriuria was determined based on a quantitative urine culture according to urine collection method used (puncture, catheter or free catch). Urine samples were collected from companion animals at teaching hospital of the Faculty of Veterinary Medicine and at private veterinary hospitals in Lisbon, Portugal.

Also, were selected 85 non-duplicate 3GC resistant - *E. coli* isolates from community-acquired urinary tract infections (CA-UTI), obtained from a Diagnostic Laboratory of the Lisbon area in 2013. Regarding the type of UTI information about epidemiological data were not available.

Isolated bacteria were stored in 20% glycerol (Sigma–Aldrich, St Louis, MO, USA) brain heart infusion broth (Biokar Diagnostics) at −80 °C for future studies. For this study, stored isolates were recovered by streaking them onto 5% sheep blood agar (bioMérieux, Marcy-l'Étoile, France) and MacConkey (Biokar Diagnostics, Allonne, France) agar plates. The plates were incubated at 37 °C for 18–20 h.

Identification and confirmation of the *E. coli* isolates was performed by PCR detection of the gadA gene (McDaniels et al., 1996).

Antimicrobial Susceptibility testing

Antimicrobial susceptibility testing was carried by the disk diffusion method on Mueller-Hinton agar as the culture medium, according to Clinical Laboratory Standards Institute (CLSI) guidelines. Veterinary CLSI breakpoints (CLSI, 2018) were used. Human CLSI breakpoints (CLSI, 2019) were used for the remaining antimicrobials. The antimicrobial agents (Oxoid, Hampshire, United Kingdom) tested were: amoxicillin 25 µg or ampicillin 10 µg, amoxicillin-clavulanate acid 30 µg, cefotaxime 30 µg, ceftazidime 30 µg, cefoxitin 30 µg, imipenem 10 µg, meropenem 10 µg, gentamicin (10 µg), tobramycin 10 µg, amikacin 30 µg, ciprofloxacin 5 µg, enrofloxacin 5 µg, norfloxacin 10 µg, nitrofurantoin 300 µg and trimethoprim/sulfamethoxazole 25 µg. ESBL production was studied in all third-generation cephalosporin-resistant isolates by the double-disk synergy test and the results were interpreted according to the to CLSI guidelines. *E. coli* ATCC 25922 from the American type Culture Collection was used as a reference strain.

Antimicrobial categories were used to characterize multidrug resistance (MDR) as previously proposed by Magiorakos et al., 2012. *E. coli* was considered as MDR when fully resistant to three or more antimicrobial categories (Magiorakos et al., 2012).

DNA extraction, sample purification and sequencing

DNA extraction was conducted using a boiling method (Féria et al., 2002). Briefly the isolates were cultured on blood agar plates for 24h. The cells were lysed by heating at 100 °C for 10 min, following centrifugation of the lysate. The supernatant was used as the source of the template DNA. For PCR amplicon sequencing, DNA purification was conducted using a NZYTech

Gel Pure Kit (NZYTech—Genes and Enzymes, Lisbon, Portugal) and sequencing was performed by Stabvida (Caparica, Portugal). Sequences were analysed using Ugene Unipro software (Unipro, Novosibirsk, Russia) and the nucleotide basic local alignment search tool (http://blast.ncbi.nlm.nih.gov/).

Molecular detection of antimicrobial resistance genes

Antimicrobial resistance genes were investigated in resistant and intermediate resistant strains.

3GC-resistant *E. coli* were screened for $bla_{\text{CTX-M}}$ genes by PCR (Edelstein et al., 2003). Isolates with $bla_{\text{CTX-M}}$ were tested for $bla_{\text{CTX-M group 1}}$, $bla_{\text{CTX-M group 2}}$ and $bla_{\text{CTX-M group 9}}$ genes with specific primers (Woodford et al., 2006) and positive amplicons were submitted to nucleotide sequencing. Cefoxitin-resistant *E. coli* were subjected to multiplex-PCR for plasmid-borne genes encoding AmpC β-lactamase (bla_{CIT}, bla_{LAT}, bla_{ACT}, bla_{MIR}, bla_{FOX}, bla_{MOX}, bla_{DHA}) using specific primers as previously described (Pérez and Hanson 2002). Positive samples for the group CIT were submitted to nucleotide sequencing after a specific PCR targeting the entire CMY-2 (Belas et al., 2014). 3GC-resistant *E. coli* negative for $bla_{\text{CTX-M-type}}$ or AmpC genes were tested for the presence of $bla_{\text{TEM-type}}$ and $bla_{\text{SHV-type}}$ ESBL genes (Féria et al., 2002).

Strains were screened by PCR for the presence of common carbapenemase genes (bla_{IMP}, bla_{OXA}, bla_{VIM}, bla_{NDM} and bla_{KPC}) as previously described (Poirel et al., 2011).

Uropathogenic *Escherichia coli* Phylogenetic typing, Pathogenicity Islands markers and Virulence genotyping determination

Phylogenetic typing was performed in all 3CG-resistant *E. coli* strains to determine the main phylogenetic groups (A, B1, B2 and D) according to the amplification of *chuA* and *yjaA* genes, and TspE4C2 fragment (Doumith et al., 2012).

Eight UPEC pathogenicity islands markers were screened by multiplex PCR assays as previously described (Sabaté et al., 2006; Bronowski et al., 2008). PCR reactions were split in three separate multiplex assays: multiplex A for PAIs III_{536}, IV_{536} and II_{CFT073}; multiplex B1 for PAIs II_{J96} and I_{536}; and multiplex B2 for PAIs II_{536}, I_{J96} and I_{CFT073}. Negative and positive controls (*E. coli* CFT073, *E. coli* 536, *E. coli* J96) were used for all PCRs (Sabaté et al., 2006; Bronowski et al., 2008).

3CG - resistant *E. coli* strains were screened by PCR for the presence of the following virulence genes: mediate adhesion (p-fimbrial adhesion genes *pap*EF operon segment), Sfa fimbriae and Afa afimbrial adhesin (*sfa* and *afa* genes, respectively), toxins (α-haemolysin *hly*A gene from the alpha-hemolysin operon, cytotoxic necrotizing factor 1 (*cnf-1* gene), aerobactin siderophore (*iuc*D gene) (Féria et al., 2002), the major pilin subunit of *E. coli* common pilus (ecpA

gene) and the bacteriocin-like genotoxin uropathogenic specific protein (*usp* gene) (Narciso et al., 2012). Negative and positive controls (*E. coli* CFT073, *E. coli* 536, *E. coli* J96 and *E. coli* KS52) were used for all PCRs. Futhermore, the hemolytic activity, associated with the expression of *E. coli* alpha-hemolysin, was evaluated on Columbia 5% sheep blood agar (bioMerieux, Marcy L'Etoile, France).

ESBL/ AmpC-producing *Escherichia coli* strains molecular typing

E. coli strains not belonging to the ST131 clonal lineage were typed by Multilocus sequence typing (MLST). Internal fragments of the seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd, mdh*, *purA*, and *recA*) were amplified by PCR using the primers and conditions described previously in https://enterobase.warwick.ac.uk/ (Wirth et al., 2006). PCR products were sequenced.

The types of sequences (ST) were submitted to the MLST database to retrieve an allelic profile and sequence type for each isolate. The ST131 clonal group, O16/O25b types and the ST131-*H*30Rx clade were identified as previously described by PCR (Banerjee et al., 2013; Colpan et al., 2013; Johnson et al., 2014).

Rapid fingerprinting using repetitive sequence-based PCR repetitive element sequence-based PCR (rep-PCR) typing method with 2 opposing primers, REP1R-I (5′-IIIICGICGICATCIGGC-3′) and REP2-I (5′-ICGICTTATCIGGCCTAC-3′) (Silva et al., 2009) was performed in the ESBL/ AmpC ß-lactamases producing *E. coli* strains that have the same MLST and the same PAI or the combination of the same PAIs. PAIs have conserved and stable 'core genome' that contains the genetic information that is required for essential cellular functions, so identification of a certain PAI or the combination of several PAIs can be characteristic for a pathogenic *E. coli* strain (Hacker and Kaper, 2000). *E. coli* strain ATCC 25922 was used for the standardisation of the rep-PCR reactions and as a positive control.

To analyze the REP DNA fingerprints obtained for *E. coli* strains was used the Bionumerics (version 6.6 Applied Maths, Sint-Martens-Latem, Belgium) software using the unweighted pair group method with arithmetic mean (UPGMA) clustering method with a tolerance of 1.0%.

Statistical Analysis

The SAS statistical software package for Windows v. 9.4 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis. The Fisher's exact test was used for comparisons between groups with a p value of 0.05.

Results

3GC-resistant *Escherichia coli* antimicrobial resistance and phylogenetic group

From a total of 330 *E. coli* strains non-duplicate recovered from companion animals with UTI, 10.6 % (*n*=35/330) were 3GC- resistant. A high proportion was resistant to ciprofloxacin (74.3%, n=26/35), norfloxacin (71.4%, (n=25/35), trimethoprim-sulfamethoxazole (71.4 %, n=25/35), gentamicin (40.0% n=14/35) and tobramycin (31.4%, n=11/35). Overall, 71.4 % (n=25/35) of the strains were MDR. Yet, no resistance to carbapenems was detected. Regarding, 3CG-resistant *E. coli* from humans-CA with UTI, these showed higher resistance frequencies against to fluoroquinolones (88.2%, *n*=75/85), trimethoprim-sulfamethoxazole (74.1 %, *n*=63/85), gentamicin (37.6% *n*=32/85) and tobramycin (49.4 %, n=42/85). Overall, 84.7 % (n=72/85) of the strains were MDR (Table1). Antimicrobial resistance frequencies of the 3GC-resistant *E. coli* strains from companion animals and non- related human (H-CA) with UTI are shown in Table 1.

Regarding phylogenetic group from companion animals 3GC - resistant *E. coli* strains belonged mainly to group-D (48.6%, n=17/35) followed by group-A (22.9%, n=8/35), group- B2 (17.1%, n=6/35) and group-B1 (11.4%, n=4/35) (Table 2). Considering phylogenetic groups 3GC resistant *E. coli* strains from human-CA with UTI belonged mainly to group-B2 (67.1%, n=57/85) followed by group-B1 (14.1%, n=12/85), group- A (12.9 %, n=11/85) and group-D (5.9 %, n=5/85) (Table 2).

Interestingly, resistance to aminoglycosides in companion animals were higher in group-D (52.9% - gentamicin) and in group-B2 (50.0 %). However, in both phylogenetic groups and in both species were detected higher frequencies of resistance to fluoroquinolones and trimethoprim-sulfamethoxazole. In companion animal strains MDR was higher in group-A and group-B1 (87.5% and 75.0%, respectively). However, in human strains MDR was higher in group-B2, group-A and group-D (91.2%, 81.8 % and 80.0%, respectively) (Supplementary S1).

The majority of 3GC resistant - *E. coli* strains belonged to the phylogenetic group-B2 in both groups (83.3% for companion animals and 94.7% for human – CA), followed by group-B1 (75.0% for companion animals and 100% for human – CA), group- A (62.5 % for companion animals and 100% for human – CA) and group-D (11.7 % for companion animals and 60.0 % for human – CA). Furthermore, statistical significance was detected in group - D between human-CA and companion animal (p =0.024). However, ESBLs - producing *E. coli* from human-CA was higher than in companion animal strains (p <0.0001) (Table 2). Regarding AmpC - producing *E. coli* strains from both groups belonged mostly to group - D (94.1% for companion animals and 40.0 % for human – CA, p <0.0001. However, AmpC - producing *E. coli* strains in companion animals was higher than in human-CA (p <0.0001) (Table 3).

In this study, different types of ESBLs were detected, especially CTX-M, indicating a high diversity of these ESBLs in UPEC strains (Table 4). However, in four 3GC - resistant *E. coli* strains the phenotype of resistance to cefoxitin was not possible be explained by all the tested AmpC β-lactamase genes, so other mechanism of resistance may be involved. Moreover, carbapenemases genes were not detected in both 3CG-resistant *E. coli* collections.

3GC *Escherichia coli* pathogenicity islands markers and virulence genotyping

Eight UPEC pathogenicity islands were screened in all 3GC *E. coli* strains and the most prevalent PAIs among strains from human CA - UTI and companion animals with UTI were PAI IV_{536} (91.8 %, *n*=78/85 and 72.3 % *n*=26/35, respectively), followed by PAI_{CFT073} (78.8 %, *n*=67/85 and 54.3 % *n*=19/35, respectively), PAI II_{CFT073} (69.4 %, *n*=59/85 and 20.0 % *n*=7/35, respectively) (Table 5). As expected, group-A and B1 has less pathogenicity island markers and virulent genes than phylogenetic group - B2 and group - D (Supplementary S2 and Supplementary S3).

All *E. coli* strains were positive for *ecpA gene* the major pilin subunit of *E. coli* common pilus for both groups ($p>0.05$). Furthermore, *pap*EF *operon segment*, *iuc*D, *hly*A and *cnf*1 were also frequent in both groups (Table 6). Although, Cytotoxic necrotizing factor-1 (*cnf*1 gene) and aerobactin siderophore (*iuc*D gene) frequencies were higher in *E. coli* from humans - CA ($p=0.012$ and $p = 0.0002$, respectively) (Table 6).

ESBL/ AmpC ß-lactamases producing *Escherichia coli* - Clonal relationship

The companion animal strains were classified into 15 STs, where as human strains were classified in 19 distinct STs (Table 7). The most common sequence types (STs) among ESBL/ AmpC ß - lactamases producing *E. coli* strains from companion animals with UTI were ST648 (*n*=11), ST131 (*n*=5), ST539 (*n*=2) and ST1775 (*n*=2).

Regarding ST648 clonal group mostly were *E. coli* CMY-2-producing (*n*=11) and one harbouring bla_{CMY-2} and $bla_{CTX-M-9}$ and includes resistance to fluoroquinolones, trimethoprim/sulfamethoxazole and other antimicrobials, such as aminoglycosides. Moreover, PAI_{IV536}- $PAI_{ICFT073}$ (*n*=5) were the combinations most detected. However, different profiles of virulence genes were found, but the most prevalent was *ecp*A-*pap*EF (*n*=7) (Supplementary S4).

In companion animals 3 *E. coli* ST131 C2/*H*30Rx clade with the $bla_{CTX-M-15}$ gene and two ST131C1/*H*30R1 (C1-non27) sub-clade with the $bla_{CTX-M-1}$ gene and one strain harbouring bla_{CMY-2} gene were detected. *E. coli* ST131 C1/*H*30R1 (C1-M27) clade with the $bla_{CTX-M-27}$ gene was not detected in companion animals with UTI and the remaining ST131 remained unclassified by this assay. For ST131 *E. coli* strains were observed that PAI_{I536}-PAI_{IIJ96}-$PAI_{ICFT073}$-PAI_{IV536}- $PAI_{IICFT073}$ (*n*=2) and $PAI_{ICFT073}$-PAI_{IV536}-$PAI_{IICFT073}$ (*n*=2), were the combinations most detected. However, different profiles of virulence genes were found (Supplementary S4).

Among phylogroup-B1 isolates belonged to ST539 (*n*=2), one harbouring *bla*$_{CMY}$-2 and one harbouring *bla*$_{CTX\text{-}M\text{-}1type}$, followed by ST533 (n=1, harbouring *bla*$_{CTX\text{-}M\text{-}15}$) and ST224 (*n*=1, harbouring *bla*$_{CTX\text{-}M\text{-}32}$). Regarding phylogroup –A, the strains belonged to unassigned ST (*n*=1, harbouring *bla*$_{CTX\text{-}M\text{-}15}$), ST609 (*n*=1, harbouring *bla*$_{CTX}$-M-32), and ST88 (*n*=1, harbouring *bla*$_{CTX\text{-}M\text{-}1}$).

The most common genotypes among ESBL/ AmpC ß-lactamases producing - *E. coli* strains from humans CA- UTI were ST131 (*n*=56), ST453 (*n*=5), ST90 (*n*=2), ST10 (*n*=2), and ST88 (*n*=2). Such as, in companion animals, human CA - UTI strains also harbored diverse ESBLs /pAmpC genes from different phylogenetic groups: group - A (*bla*$_{CTX\text{-}M\text{-}32}$ *n*=4, *bla*$_{CTX\text{-}M\text{-}1}$ *n*= 3, *bla*$_{CTX\text{-}M\text{-}14}$ *n*=1, *bla*$_{CTX\text{-}M\text{-}27}$ n=1, *bla*$_{CTX\text{-}M\text{-}15}$ n=1 and *bla*$_{CTX\text{-}M\text{-}9like}$ n=1), group - B1 (*bla*$_{SHV\text{-}12}$ n=1, *bla*$_{CTX\text{-}M\text{-}14}$ n= 4, *bla*$_{CTX\text{-}M\text{-}1}$ n=4, *bla*$_{CTX\text{-}M\text{-}15}$ n= 2 and *bla*$_{CTX\text{-}M\text{-}2}$ n=1), group - D (*bla*CTX-M-15 n=2, *bla*$_{CTX\text{-}M\text{-}14}$ n=1 and *bla*$_{CMY\text{-}2}$ n=2). Among group - B2 *E. coli* strains belonged mainly to the ST131-C2/H30Rx clade with the *bla*$_{CTX\text{-}M\text{-}15}$ gene (47.1 %, *n*=40/85). Also, *E. coli* ST131-C1-M27 sub-clade with the *bla*$_{CTX\text{-}M\text{-}27}$ gene was detected in 9.1% (*n*=5/55).

About 18.8 % (*n*=16/55) of the ESBL/ AmpC ß-lactamases producing - *E. coli* strains were ST131- C1/*H*30R1-non-M27 clade and 2 harbouring *bla*$_{CTX\text{-}M\text{-}1}$ gene, 1 harbouring *bla*$_{CTX\text{-}M\text{-}15like}$ gene, 1 harbouring *bla*$_{CTX\text{-}M\text{-}32}$ gene, 8 harbouring *bla*$_{CTX\text{-}M\text{-}9}$ group gene, 2 harbouring *bla*$_{CTX\text{-}M\text{-}9like}$ gene and 1 harbouring *bla*$_{CTX\text{-}M\text{-}14}$ gene. Furthermore, in this study also, the clone O16-H5-ST131 (clade A) with the *bla*$_{CTX\text{-}9like}$ was detected. Furthermore, three strains from the pandemic clone O25b:H4-B2-ST131 were AmpC-producers with *bla*$_{CMY\text{-}2}$ gene (Supplementary S5).

Regardless, ST131 *E. coli* strains from human CA-UTI the most frequent combinations found were PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$ (*n*=27) and PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$ (*n*=23).

The distribution of the different virulence genes showed that the most prevalent profiles in strains from humans-CA belonged to group-B2 were *ecp*A-*iuc*D (*n*=17), *ecpA-papEF-sfaDE-hlyA-cnf1-iucD* (*n*= 15) and *ecpA-papEF-hlyA-cnf1-iucD* (*n*=13) (Supplementary S5).

Among group - D and group - B1, PAI$_{ICFT073}$-PAI$_{IV536}$ (*n*=2 and *n*=8, respectively) was the combination most frequent. For group-D and group-B1 was the profile *ecp*A-*iuc*D (*n*=2 and *n*=5, respectively). Group - B1 and group - D had the identical pathogenic islands marker and virulence genes profiles. The presence of ExPEC - associated virulence genes is shown in Supplementary S5.

The ESBL/AmpC ß-lactamases producing *E*. *coli* STs were compared phylogenetically based on a tree using goeburst, based on phyloviz software. Putative founder STs in both human and companion animals *E*. *coli* belonged to clonal complex (CC) 131, CC 648 and CC23. ESBL/AmpC ß-lactamases producing *E*. *coli* from companion animals and humans (CA) with UTI shared two MDR high-risk clonal lineages: the ST131 and ST648. Moreover, two other clonal lineages were shared by companion animals and human, ST88 and ST354 (Table 7, Figure1,

Supplementary Tables S4 and S5). Regarding rep-PCR confirmed that ESBL/AmpC ß-lactamases producing *E. coli* from companion animals and humans (CA) with UTI with the same STs, identified by MLST and with the same combination of PAIs demonstrated identical rep-PCR fingerprints (Figure 2).

Discussion

The high frequency of resistance to antimicrobials that are categorised as critically important in human medicine, amoxicillin–clavulanic acid, 3GCs, fluoroquinolones and aminoglycosides that are normally used in veterinary medicine and human medicine is worrisome for public health.

MDR was displayed by a high percentage of 3GC-resistant *E. coli* strains from companion animals and non-related humans from the community (CA) with UTI.

In this study a large diversity of ESBL/AmpC ß-lactamases producing *E. coli* from companion animals and non-related humans (H-CA) with UTI were identified. However, in companion animals was frequently associated with the presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CMY-2}}$ genes, and in humans (CA) with $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-1}}$. Furthermore, CMY-2-producing *E. coli* strains in companion animals was higher than in human-CA. In a study performed in UK, the authors also found a high prevalence of CMY-2- producing *E. coli* strains from dogs with UTI (Wagner et al., 2014). Furthermore, the majority of CMY-2-producing *E. coli* strains belonged to phylogenetic group D, which is consistent with previous study performed in United States (Liu et al., 2016).

The majority of 3GC-resistant *E. coli* strains belonging to the phylogenetic group B2 had the majority of the PAI markers. The association of group B2 and several PAI markers has previously been reported among UPEC strains (Sabaté et al., 2006). However, A, B1, and D phylogenetic groups of UPEC strains have less PAIs markers than other groups as described before by other authors (Sabaté et al., 2006; Mateus et al., 2013) The presence of virulence factors is linked to PAI markers. Furthermore, the most frequent PAI combination pattern was related to those strains containing PAI_{IV536} and $PAI_{ICFT073}$ markers These PAI markers contain iron uptake system encoding genes (such as, *iuc*D gene) and seem to be important for effective colonization and the fitness of UPEC strains throughout the urinary tract, but also, fimbrial adhesins, high prevalence of fimbrial adhesin-encoding genes (such as, *papEF operon segment*) from *E. coli* isolates in patients diagnosed with UTI confirms that these structures are necessary to cause an UTI (Sarowska et al., 2019). Futhermore, $PAI_{ICFT073}$ marker also, carries the toxin hemolysin A (*hly*A gene), that is responsible for the creation of pores in membranes and cell lysis (Sarowska et al., 2019). These results were consistent with other studies, which compared *E. coli* pathogenicity background (Féria et al., 2002; Sabaté et al., 2006; Tramuta et al., 2011; Mateus et al., 2013; Toval et al.2014).

E. coli common pilus (*ecpA* gene) was detected in all isolates from both groups. This pilus playing a role in the adhesion of *E. coli* to the intestinal epithelium and the colon functions are act as a reservoir that can be responsible for the recurrent UTIs and allows some mechanisms to evade the immune system from their hosts. Also, is associated to both pathogenic and commensal *E. coli* strains. The *ecp*A gene is kept in intestine and can confer protection to antimicrobials, which can reduce the therapeutic options and the dissemination of ESBLs. (Rendón et al., 2007; Narciso et al. 2012). Moreover, in this study only in one *E. coli* ST131 (from a dog obtained in 2015) was detected the uropathogenic-specific protein gene (*usp*). This protein gene is a genotoxin active against mammalian cells associated with isolates from pyelonephritis, prostatitis, bacteremia of urinary tract origin and can induces characteristic of apoptosis. It has been proposed that usp provide immunity to the producer and that it also enhances infectivity in the urinary tract (Nipič et al., 2013; Crnigoj et al, 2014). More studies are needed to understand the importance of this specific protein gene in urinary tract infections.

In this study two MDR high-risk clonal lineages, the ST131 and ST648, and also ST88 were shared between companion animals and humans with UTI and also had identical rep-PCR fingerprints.

MLST has the advantage of being comparable worldwide, unambiguous and highly reproducible (Sabat et al., 2013). In this study rep-PCR was also performed as an alternative to Pulsed-Field Gel Electrophoresis (PFGE). The rep-PCR has also a good discriminatory power, is less time consuming, less costly than PFGE and it presents a good correlation with the PFGE results (Jonas et al., 2003). Given all these factors, MLST and rep-PCR were the selected techniques for analysing the data of this study. Moreover, the *E. coli* strains from humans and companion animals were collected in different years, so the relationship among these strains should be further investigated by Whole-genome sequencing.

Interestedly, *E. coli* ST88 clone are associate with poultry and broiler meat origins, which suggested that animals from farms are reservoirs for this type of *E. coli* that causes extraintestinal disease in humans and companion animals (Day et al., 2016; van Hoek et al., 2018; Borges et al., 2019).

The ST131 is the predominant clone driving the spread of cephalosporin and fluoroquinolone resistance and have disseminated to various animal species, including humans, poultry, pigs, and companion animals. (Nicolas-Chanoine et al., 2014). Furthermore, is the mostly responsible for the global dissemination of extended-spectrum beta-lactamase-producing *E. coli* worldwide and the most frequent in *E. coli* urinary tract infection in humans (Nicolas-Chanoine et al., 2014).

The $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ genes are the most common types of ESBLs among human strains with UTI, though $bla_{\text{CTX-M-14}}$ gene has mainly been reported in Japan, Asian countries, Canada and Spain (Peirano et al., 2010; Zong and Hu, 2013). The ST131 clone harbouring

bla$_{CTX-M-15}$ or *bla*$_{CTX-M-14}$ has also been detected among companion animals in many countries (Ewers et al., 2010; Pomba et al., 2014; Belas et al., 2019). In Japan, Matsumura et al. (2016) between 2005 and 2010 found the bla$_{CTX-M-14}$ gene to be the most common, followed by *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-2}$. They recently reported on the global emergence and increased prevalence of the *E. coli* ST131 sub- clade (C1/*H*30R1, named the C1-M27) (Matsumura et al., 2016). The *bla*$_{CTX-M-27}$, a single-nucleotide variant of *bla*$_{CTX-M-14}$ has been increasingly identified among the *E. coli* strains from human and companion animals with UTI in United States, Asian and European countries. (Harada et al., 2012; Matsumura et al., 2016; Bevan et al., 2017) and in clinical *E. coli* strains from companion animals in France (Melo et al., 2019). Moreover, transmission of the *E. coli* ST131 clone among family members and companion animals has been documented, increasing the risk in human of causing severe infections difficult to be treated (Johnson et al., 2009a).

The results of this study suggest that the C2/*H*30Rx subclade is a prevalent clone in the Lisbon area in Portugal in human -CA with UTI and that the majority of these isolates lack different types of ESBL genes. Nevertheless, the clone has previously been demonstrated as being highly virulent and, when MDR, it may have a direct impact in the management of community associated UTI in both humans and animals. This clone is normally associated with complicated UTIs (Campos et al., 2018). However, to the best of our knowledge, in this study we have the first description of *E. coli* O25b:H4-ST131 harbouring *bla*$_{CMY-2}$ gene and also, the first description of the subclade C1-M27 in human in the community with UTI in Portugal. Yet, C1-M27 subclade has been detected in faecal samples of healthy humans in the north of Portugal (Rodrigues et al., 2016). Moreover, *E. coli* O25b:H4-ST131 harbouring *bla*$_{CMY-2}$ gene has been rarely described (Day et al., 2016; Hansen et al., 2016).

In previous studies clinical *E. coli* showed that the O16-H5-ST131 clone (clade A) is globally distributed (Johnson et al., 2014). Yet, in this study to best of our knowledge we have the first description of the O16:H5-ST131 clone harbouring *bla*$_{CTX-9like}$ in a human-CA with UTI in Portugal. In this study it is noteworthy that ST648 strains were strongly associated with *bla*$_{CMY-2}$ gene and also combines MDR and virulence. Furthermore, further studies for ST648 strains are important to be performed in companion animals, because the transmission of these clones to the humans can occur by direct contact or by environmental contamination.

Conclusion

Our findings are of critical relevance, as they show companion animals and humans as reservoirs of pandemic clones, especially *E. coli* ST131-C2/*H*30Rx (*bla*$_{CTXM-15}$) and ST648 harbouring CMY-2. Furthermore, *E. coli* ST10 and ST410 other important pandemic lineages were among the STs detected in humans CA-UTI (Mathers et al., 2015; Campos et al., 2018). Yet, we did not find these ST types in companion animals with UTI.

The inappropriate use of antimicrobials in Human and Veterinary medicine also plays an important role in this complex problem. As such, antimicrobial use should be reduced to a minimum and alternative approaches should be used to limit the spread of antimicrobial resistance in animals and humans (Bélanger et al., 2011). This study allows to understand some aspects of the dissemination of ESBLs/AmpC- producing *E. coli* in Lisbon area, which is important step for developing strategies to prevent the propagation of high-risk clones. In a One-Health perspective, the collaboration between Veterinary medicine and Human medicine is needed to characterize the occurrence and routes of dissemination of these high-risk clones.

Considering that companion animals with UTI are generally treated at home by the owners, measures should be implemented to avoid the spread of these bacteria to the environment.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author contributions

AB performed phenotypic and molecular characterization of companion animal and human isolates, analyzed and interpreted the data, and wrote the manuscript. AB and LTG performed statistical analysis. JM performed rep-PCR. JAC revised the statistical. CP planned and coordinated the study and checked the manuscript. All authors revised and approved the final version of the manuscript.

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Table 1: Antimicrobial resistance of third-generation cephalosporins - resistant *Escherichia coli* strains resistant from companion animals and human -CA with UTI.

Antimicrobials	Companion animal (N =35)a % (n)	Human- CA (N =85)a % (n)	*P* value
Ampicillin/ Amoxicillin	100% (*n* = 35)	100% (*n* =85)	N.s.
Amoxicillin/clavulanate	**77.1% (*n* = 27)**	27.1% (*n* =23)	<0.0001
Cefoxitin	**62.9% (*n* = 22)**	8.2% (*n* =7)	<0.0001
Cefotaxime	91.4% (*n* =32)	**100% (*n* =85)**	0.023
Ceftazidime	62.8% (*n* =22)	**42.4% (*n* =36)**	0.047
Imipenem	0.0 % (*n* = 0)	0.0% (*n* =0)	N.s.
Meropenem	0.0 % (*n* = 0)	0.0% (*n* =0)	N.s.
Ciprofloxacin	74.3% (*n* =26)	**88.2% (*n* =75)**	0.096
Norfloxacin	71.4% (*n* =25)	**88.2% (*n* =75)**	0.033
Nitrofurantoin	5.7% (*n* =2)	2.4% (*n* =2)	N.s
Gentamicin	40.0% (*n* =14)	37.6% (*n* =32)	0.838
Amikacin	5.7% (*n* =2)	10.6% (*n* =9)	0.506
Tobramycin	31.4% (*n* =11)	49.4% (*n* =42)	0.105
Trimethoprim/sulfamethoxazole	71.4% (*n* =25)	74.1% (*n* =63)	0.822
Multidrug resistant	71.4 % (*n* =25)	84.7% (*n* =72)	0.125

Legend: %R, percentage of resistant strains; Human-CA, human community-acquired UTI. [a]The number shown is the total number of strains tested. * p value < 0.05 statistically significant. N.s. not significant to calculate.

Table 2: Third generation cephalosporins resistant – *Escherichia coli* strains phylogenetic group from companion animals and human - CA with UTI.

Phylogenetic group	Companion animal (*N* =35)[a] % (*n*)	Human - CA (*N* =85)[a] % (*n*)	*P* value
Group A	22.9 % (*n*=8)	12.9 % (*n*=11)	0.187
Group B1	11.4 % (*n*=4)	34.3 % (*n*=12)	0.777
Group B2	17.1% (*n*=6)	**67.0 % (*n*=57)**	<0.0001
Group D	**48.6 % (*n*=17)**	5.9 % (*n*=5)	<0.0001

Legend: Human-CA, human community-acquired UTI. [a]The number shown is the total number of strains tested. * p value < 0.05 statistically significant.

Table 3: ESBLs/AmpC -producing *Escherichia coli* strains by phylogenetic group from companion animals and human-CA with UTI.

Phylogenetic group	Companion animal (*N* = 35)[a]		Phylogenetic group	Human - CA (N = 85)[a]		*P* value	*P value*
	ESBLs genes % (*n*)	AmpC genes % (*n*)		ESBLs genes % (*n*)	AmpC genes % (*n*)	ESBLs genes	AmpC genes
A (*n*=8)	62.5 % (*n*=5)	0.0 % (*n*=0)	A (*n*=11)	100 % (*n*=11)	0.0 % (*n*=0)	0.057	N.s
B1 (*n*=4)	75.0 % (*n*=3)	50.0 % (*n*=2)	B1 (*n*=12)	100 % (*n*=12)	0.0 % (*n*=0)	0.250	0.050
B2 (*n*=6)	83.3 % (*n*=5)	33.3 % (*n*=2)	B2 (*n*=57)	94.7 % (*n*=54)	5.3 % (*n*=3)	0.337	0.067
D (*n*=17)	11.7 % (*n*=1)	94.1% (*n*=16)	D (*n*=5)	**60.0 % (*n*=3)**	40.0 % (*n*=2)	0.024	<0.0001
Overall	38.9 % (*n*=14)	**57.1 % (*n*=20)**	Overall	**94.1 % (*n*=80)**	5.9 % (*n*=5)	<0.0001	<0.0001

Legend: % percentage of strains; human community-acquired UTI; [a]The number shown is the total number of strains tested; ESBLs- extended-spectrum β-lactamases; AmpC- AmpC β-lactamases; * p value < 0.05 statistically significant. N.s. not significant to calculate.

Table 4: ESBLs/AmpC - producing *Escherichia coli* strains from companion animals and human-CA with UTI.

ESBL/AmpC genes	Companion animal (N = 35)[a] % (n)	Human - CA (N = 85)[a] %(n)
bla$_{SHV-12}$	0.0 % (*n*=0)	1.2 % (*n*=1)
bla$_{CTX-M-1}$	5.7 % (*n*=2)	10.6 % (*n*=9)
bla$_{CTX-M-1-type}$	5.7 % (*n*=2)	0.0 % (*n*=0)
bla$_{CTX-M-15}$	20.0 % (*n*=7)	54.2 % (*n*=46)
bla$_{CTX-M-15- type}$	0.0 % (n=0)	1.2 % (*n*=1)
bla$_{CTX-M-32}$	8.6 % (*n*=3)	5.9 % (*n*=5)
bla$_{CTX-M-9}$	2.9 % (*n*=1)	0.0 % (*n*=0)
bla$_{CTX-M-9-type}$	0.0 % (*n*=0)	4.7 % (*n*=4)
bla$_{CTX-M-14}$	0.0 % (*n*=0)	8.2 % (*n*=7)
bla$_{CTX-M-27}$	0.0 % (*n*=0)	7.1 % (*n*=6)
bla$_{CTX-M-2group}$	0.0 % (*n*=0)	1.2% (*n*=1)
bla$_{CMY-2}$	57.1% (*n*=20)	5.9 % (*n*=5)

Legend: % percentage of strains; human community-acquired UTI; aThe number shown is the total number of strains tested; ESBLs- Extended-spectrum β-lactamases; AmpC- AmpC β-lactamases * p value < 0.05 statistically significant.

Table 5 - Frequency of pathogenicity islands markers (PAIs) among third-generation cephalosporins resistant *Escherichia coli* strains from companion animals and human-CA with UTI.

PAIs	Companion animal (Total *N*=35)[a] % (n)	Human - CA (Total *N* =85)[a] % (n)	*P* value
PAI$_{IJ96}$	0.0 % (*n*=0)	0.0% (*n*=0)	N.s.
PAI$_{IIJ96}$	11.4 % (*n*=4)	**41.2 % (*n*=35)**	0.004
PAI$_{I536}$	8.6 % (*n*=3)	**40.0 % (*n*=34)**	0.0005
PAI$_{II536}$	22.9 % (*n*=8)	35.3 % (*n*=30)	0.203
PAI$_{III536}$	0.0 % (*n*=0)	0.0% (*n*=0)	N.s.
PAI$_{IV536}$	72.3 % (*n*=26)	**91.8 %** (*n*=78)	0.017
PAI$_{ICFT073}$	54.3 % (*n*=19)	**78.8 %** (*n*=67)	0.013
PAI$_{IICFT073}$	20.0 % (*n*=7)	**69.4 %** (*n*=59)	<0.0001

Legend: Total *N*, total sample number; n, number of strains; Human-CA, human community-acquired UTI. [a]The number shown is the total number of strains tested * p value < 0.05 statistically significant. N.s. not significant to calculate.

Table 6 - Frequency of virulence genes among third-generation cephalosporins resistant *Escherichia coli* strains to from companion animals and humans-CA with UTI.

Target gene product/function	Target gene	Companion animal (Total *N*=35 [a])	Human - CA (Total *N* =85 [a])	*P* value
Pap fimbriae	*papEF operon segment*	45.7 % (*n*=16)	49.4 % (*n*=42)	0.841
Sfa fimbriae	*sfa*	20.0 % (*n*=7)	20.0 % (*n*=17)	N. s
Afa afimbrial adhesin	*afa*	2.9 % (*n*=1)	9.4 % (*n*=8)	0.281
alpha-hemolysin operon	*hlyA*	40.0 % (*n*=14)	42.4 % (*n*=36)	0.841
Cytotoxic necrotizing factor-1	*cnf1*	17.1 % (*n*=6)	**41.2 % (*n*=35)**	0.012
aerobactin siderophore	*iucD*	48.6 % (*n*=17)	**83.5 % (*n*=71)**	0.0002
E. coli common pilus	*ecpA*	100 % (*n*=35)	100 % (*n*=85)	N.s.
uropathogenic specific protein	*usp*	2.9 % (*n*=1)	0.0 % (*n*=0)	0.292

Legend: Total N, total sample number; n, number of strains; aThe number shown is the total number of strains tested; Human-CA, human community-acquired UTI.* p value < 0.05 statistically significant. N.s. not significant to calculate.

Table 7 : Distribuition of multiloccous sequence types (ST) of ESBLs/AmpC –producing *Escherichia coli* strains obtained in Portugal from companion animals and humans from community-acquired with urinary tract infections.

phylogroup	Sequence Type	Clonal Complex	β-lactamase type (ESBLs/*p*AMPc)	Specie (n)
A	ST10	10	*bla*$_{CTX-M-1}$	Human (2)
A	ST23	23	*bla*$_{CTX-M-32}$	Cat (1)
A	ST88	23	*bla*$_{CTX-M-1}$	Dog (1)
			bla$_{CTX-M-15}$	Human (1)
			bla$_{CTX-M-32}$	Human (1)
A	ST90	23	*bla*$_{CTX-M-9like}$	Human 1)
			bla$_{CTX-M-27}$	Human (1)
A	ST167	10	*bla*$_{CTX-M-32}$	Human (1)
A	ST540	–	*bla*$_{CTX-M-32}$	Human (1)
A	ST609	46	*bla*$_{CTX-M-32}$	Dog (1)
A	ST617	10	*bla*$_{CTX-M-1}$	Human (1)
A	ST5257	–	*bla*$_{CTX-M-32}$	Human (1)
A	ST6023	–	*bla*$_{CTX-M-14}$	Human (1)
A	Unassigned ST*	–	*bla*$_{CTX-M-15}$	Dog (1)
B1	ST58	155	*bla*$_{CTX-M-1}$	Homem (2)
B1	ST224	–	*bla*$_{CTX-M-32}$	Cat (1)
B1	ST453	86	*bla*$_{SHV-12}$	Human (1)
			bla$_{CTX-M-1}$	Human (1)
			bla$_{CTX-M-15}$	Human (1)
			bla$_{CTX-M-14}$	Human (3)
B1	ST533	–	*bla*$_{CTX-M-15}$+ *bla*$_{CMY-2}$	Dog (1)
B1	ST539	–	*bla*$_{CTX-M-1like}$	Dog (1)
			bla$_{CMY-2}$	Cat (1)
B1	ST847	–	*bla*$_{CTX-M-14}$	Human (1)
B1	ST1196	–	*bla*$_{CTX-M-1}$	Human (1)
B1	ST1725	–	*bla*$_{CTX-M-15}$	Human (1)
B1	ND	–	*bla*$_{CTX-M-2group}$	Human (1)
B2	ST131	131	*bla*$_{CTX-M-1}$	Cat (1), Human (2)
			bla$_{CTX-M-15}$	Dog (2), Cat (2), Human (42)
			bla$_{CTX-M-32}$	Human (1)
			bla$_{CTX-M-9like}$	Human (3)
			bla$_{CTX-M-14}$	Human (1)
			bla$_{CTX-M-27}$	Human (5)
			bla$_{CMY-2}$	Dog (1), Human (2)
B2	ST372	–	*bla*$_{CTX-M-15}$	Dog (1)
D	ST57	350	*bla*$_{CMY-2}$	Dog (1)
D	ST117	–	*bla*$_{CTX-M-15}$	Human (1)
D	ST354	354	*bla*$_{CTX-M-14}$	Human (1)
			bla$_{CMY-2}$	Dog (1)

D	ST405		405	*bla*$_{CMY-2}$	Dog (1)
D	ST410		–	*bla*$_{CTX-M-15}$	Human (1)
D	ST648		648	*bla*$_{CTX-M-9}$ + *bla*$_{CMY-2}$	Cat (1)
				bla$_{CMY-2}$	Dog (4), Cat (7), Human (1)
D	ST778		38	*bla*$_{CMY-2}$ (1)	Human
D	ST1775		–	*bla*$_{CMY-2}$ (2)	Dog
D	ST3258	–		*bla*$_{CMY-2}$ (1)	Dog

Legend: ND- not done; - : not applicable; * New ST allelic profile - Marques *et al*., 2017.

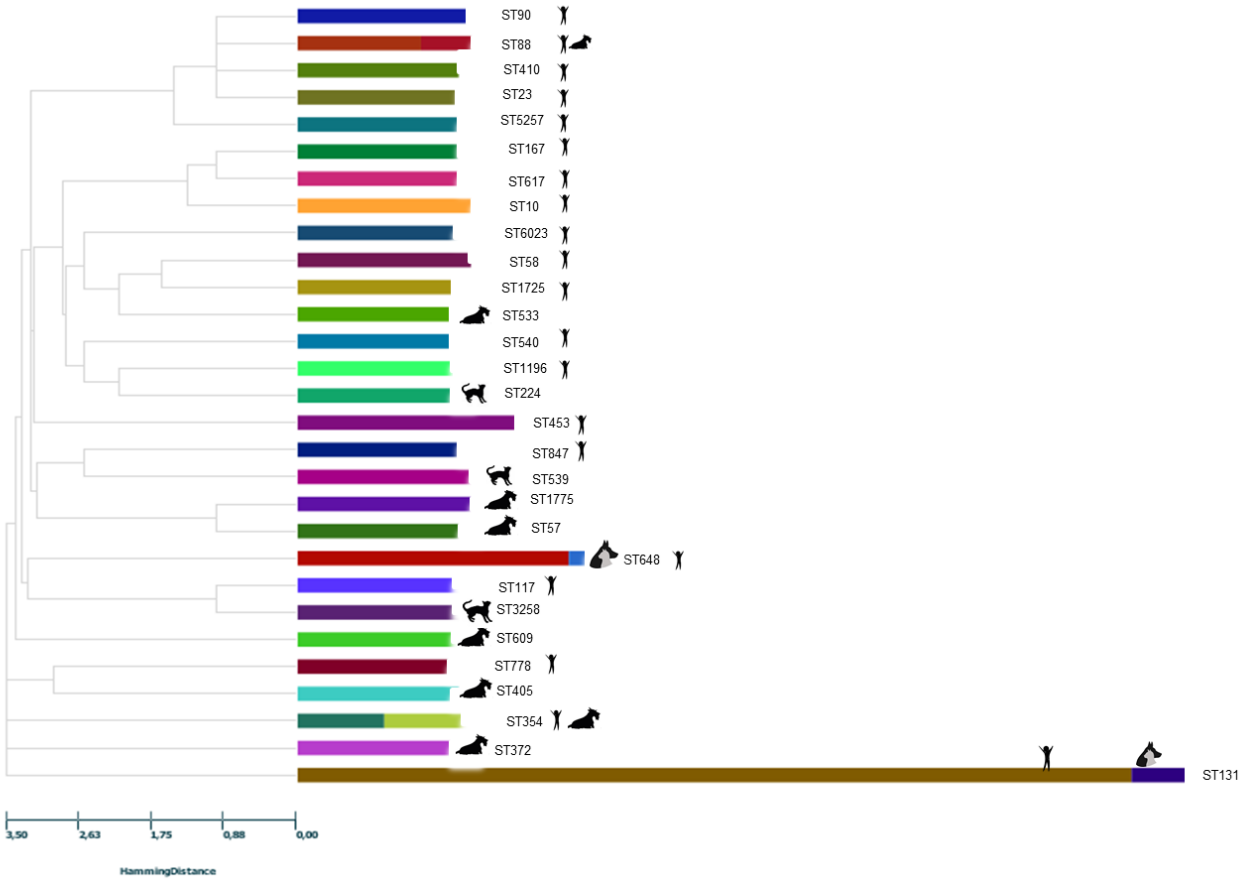

Figure 1: Distribuition of multiloccous sequence types (ST) of ESBLs/AmpC –producing *Escherichia coli* strains obtained in Portugal from companion animals and humans from community-acquired with urinary tract infections, using goeburst, based on phyloviz software.

Legend: Each ST is represented by a bar and the size is proportional to the number of strains detected.

- Human; - Dog; Cat ; - dogs and cats.

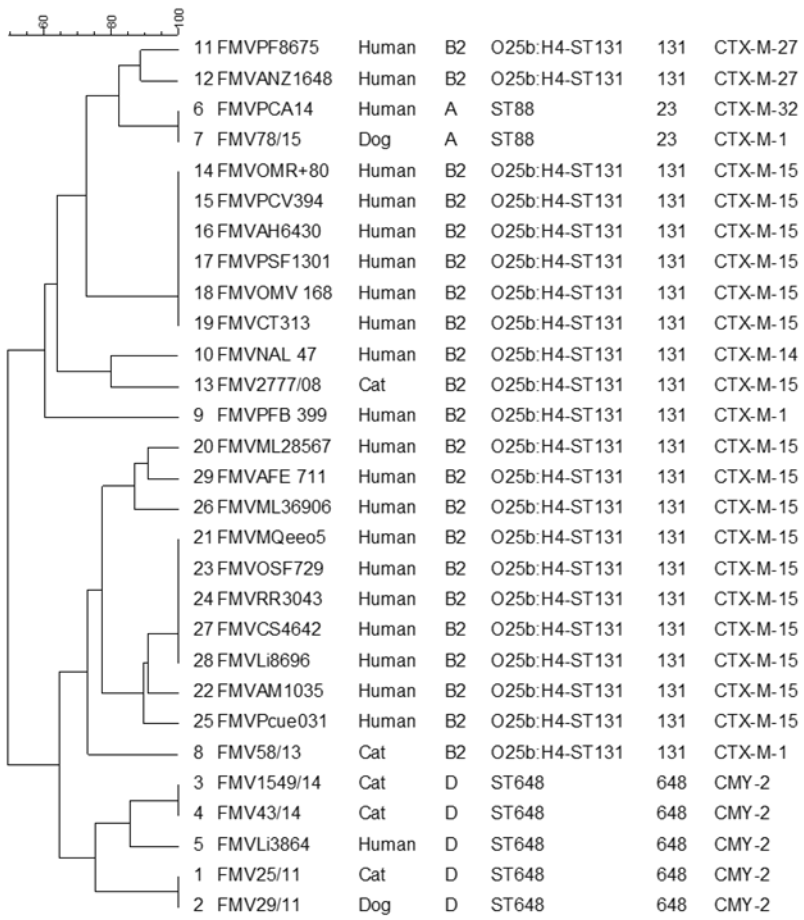

Figure 2: Dendrogram showing the relatedness by rep-PCR of *E. coli* strains from companion animals with UTI and humans-CA with UTI

Supplementary Table 1: Antimicrobial resistance of third-generation cephalosporinases - resistant *Escherichia coli* strains by phylogenetic group from companion animals and human - CA with UTI.

Antimicrobials	Companion animal (*N* =35)[a]					Human - CA (*N* =85)[a]				
	Overall (*n*=35) %R	A (*n*=8) %R	B1 (*n*=4) %R	B2 (*n*=6) %R	D (*n*=17) %R	Overall (*n*=85) %R	A (*n*=11) %R	B1 (*n*=12) %R	B2 (*n*=57) %R	D (*n*=5) %R
Ampicillin/ Amoxicillin	100% (*n* = 35)	100% (*n* = 8)	100% (*n* = 4)	100% (*n* = 6)	100% (*n* = 17)	100% (*n* =85)	100% (*n* =11)	100% (*n* =12)	100% (*n* =57)	100% (*n* =5)
Amoxicillin/clavulanate	77.1% (*n* = 27)	62.5% (*n* = 5)	75.0% (*n* = 3)	50.0% (*n* = 3)	94.1% (*n* = 16)	27.1% (*n* =23)	9.1% (*n* =1)	16.7% (*n* =2)	31.6% (*n* =18)	40.0% (*n* =2)
Cefoxitin	62.9% (*n* = 22)	37.5% (*n* = 3)	50.0% (*n* = 2)	33.3% (*n* = 2)	88.2% (*n* = 15)	8.2% (*n* =7)	0.0% (*n* =0)	0.0% (*n* =0)	8.8% (*n* =5)	40.0% (*n* =2)
Cefotaxime	91.4% (*n* =32)	87.5% (*n* = 7)	100% (*n* =4)	83.3% (*n* =5)	94.1% (*n* =16)	100% (*n* =85)	100% (*n* =11)	100% (*n* =12)	100% (*n* =57)	100% (*n* =5)
Ceftazidime	62.8% (*n* =22)	37.5% (*n* = 3)	75.0% (*n* = 3)	50.0% (*n* =3)	76.5% (*n* =13)	42.4% (*n* =36)	36.4% (*n* =4)	16.7% (*n* =2)	47.4% (*n* =27)	60% (*n* =3)
Imipenem	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)

Meropenem	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* = 0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)	0.0% (*n* =0)
Ciprofloxacin	74.3 % (*n* =26)	75.0% (*n* =6)	100% (*n* =4)	66.7% (*n* =4)	70.6% (*n* =12)	88.2% (*n* =75)	72.7% (*n* =8)	66.7% (*n* =8)	96.5% (*n* =55)	80.0% (*n* =4)
Norfloxacin	71.4% (*n* =25)	62.5% (*n* =5)	75.0% (*n* =3)	66.7% (*n* =4)	76.5% (*n* =13)	88.2% (*n* =75)	72.7% (*n* =8)	66.7% (*n* =8)	96.5% (*n* =55)	80.0% (*n* =4)
Nitrofurantoin	5.7% (*n* =2)	12.5% (*n* =1)	0.0% (*n* =0)	0.0% (*n* =0)	5.9% (*n* =1)	2.4% (*n* =2)	0.0% (*n* =0)	0.0% (*n* =0)	3.5% (*n* =2)	0.0% (*n* =0)
Gentamicin	40.0% (*n* =14)	12.5% (*n* = 1)	25.0% (*n* =1)	50.0% (*n* =3)	52.9% (*n* =9)	37.6% (*n* =32)	27.3% (*n* =3)	8.3% (*n* =1)	47.4% (*n* =27)	20.0% (*n* =1)
Amikacin	5.7% (*n* =2)	0.0% (*n* =0)	0.0% (*n* =0)	16.7% (*n* =1)	5.9% (*n* =1)	10.6% (*n* =9)	0.0% (*n* =0)	0.0% (*n* =0)	15.8% (*n* =9)	0.0% (*n* =0)
Tobramycin	31.4% (*n* =11)	12.5% (*n* =1)	25.0% (*n* =1)	50.0% (*n* =3)	35.3% (*n* =6)	49.4% (*n* =42)	36.4% (*n* =4)	0.0% (*n* =0)	64.9% (*n* =37)	20.0% (*n* =1)
Trimethoprim/sulfamethoxazole	71.4% (*n* =25)	100% (*n* =8)	50.0% (*n* =2)	33.3% (*n* =2)	76.5% (*n* =13)	74.1% (*n* =63)	81.8% (*n* =9)	75.0% (*n* =9)	71.9% (*n* =41)	80.0% (*n* =4)
Multidrug resistant	71.4 % (*n* =25)	87.5 % (*n* =7)	75.0 % (*n* =3)	66.7 % (*n* =4)	64.7 % (*n* =11)	84.7% (*n* =72)	81.8 % (*n* =9)	58.3 % (*n* =7)	91.2% (*n* =52)	80.0 % (*n* =4)

Legend: %R, percentage of resistant isolates; Human-CA, human community-acquired UTI. [a]The number shown is the total number of isolates tested.

Supplementary Table 2: Frequency of pathogenicity islands markers (PAIs) by phylogenetic group, among third-generation cephalosporins – resistant *Escherichia coli* strains to from companion animals and humans - CA with UTI.

Pathogenicity Islands markers (PAIs)									
phylogroup	**PAIs**	**PAI I_{J96}**	**PAI II $_{J96}$**	**PAI I $_{536}$**	**PAI II $_{536}$**	**PAI III $_{536}$**	**PAI IV $_{536}$**	**PAI I $_{CFT073}$**	**PAI II $_{CFT073}$**
	% (n)	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**	**% (n)**
Group A									
Human-CA (*n*=11)[a]	63.6 % (*n*=7)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	63.6 % (*n*=7)	18.2 % (*n*=2)	0.0 % (*n*=0)
Companion animal (*n* = 8)[a]	50.0 % (*n*=4)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	50.0 % (*n*=4)	0.0 % (*n*=0)	0.0 % (*n*=0)
P value	0.657	N.s	N.s	N.s	N.s	N.s	0.058	0.164	N.s
Group B1									
Human-CA (*n*=12)[a]	**75.0 % (*n*=9)**	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	**75.0 % (*n*=9**)	66.7 % (*n*=8)	0.0 % (*n*=0)
Companion animal (*n*=4)[a]	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)
P value	0.019	N.s	N.s	N.s	N.s	N.s	0.019	0.077	N.s
Group B2									
Human-CA (*n*=57)[a]	100 % (*n*=57)	0.0 % (*n*=0)	57.9 % (*n*=33)	57.9 % (*n*=33)	50.9 % (*n*=29)	0.0 % (*n*=0)	100 % (*n*=57)	94.7 % (*n*=54)	94.7 % (*n*=54)
Companion animal (*n*=6)[a]	100 % (*n*=6)	0.0 % (*n*=0)	66.6 % (*n*=4)	33.3 % (*n*=2)	16.7 % (*n*=1)	0.0 % (*n*=0)	100 % (*n*=6)	100 % (*n*=6)	100 % (*n*=6)
P value	N.s	N.s	N.s	0.393	0.199	N.s	N.s	N.s	N.s
Group D									
Human-CA (*n*=5)[a]	100 % (*n*=5)	0.0 % (*n*=0)	**40.0 % (*n*=2)**	20.0 % (*n*=1)	20.0 % (*n*=1)	0.0 % (*n*=0)	100 % (*n*=5)	60.0 % (*n*=3)	20.0 % (*n*=1)
Companion animal (*n*=17)[a]	100 % (*n*=17)	0.0 % *n*=0)	0.0 % (*n*=0)	5.9 % (*n*=1)	41.2 % (*n*=7)	0.0 % (*n*=0)	94.2 % (*n*=16)	76.5 % (*n*=13)	5.9 % (*n*=1)
P value	N.s	N.s	0.043	0.411	0.369	N.s	N.s	0.585	0.411

Legend: % percentage of strains; human community-acquired UTI; [a]The number shown is the total number of strains tested; * p value < 0.05 statistically significant. N.s. not significant to calculate.

Supplementary Table 3: Frequency of virulence genes by phylogenetic group, among third-generation cephalosporins-resistant *Escherichia coli* strains from companion animals and humans-CA with UTI.

Virulence target genes (VFs)									
phylogroup	**Strains with VFs % (n)**	***papEF*** **% (n)**	***sfa*****DE** **% (n)**	***afa*****BC** **% (n)**	***hly*****A** **% (n)**	***cnf*****1** **% (n)**	***iucD*** **% (n)**	***ecpA*** **% (n)**	***usp*** **% (n)**
Group A									
Human-CA (*n*=11)[a]	100 % (*n*=11)	27.3 % (*n*=3)	0.0 % (*n*=0)	9.1 % (*n*=1)	0.0 % (*n*=0)	0.0 % (*n*=0)	18.2 % (*n*=2)	100 % (*n*=11)	0.0 % (*n*=0)
Companion animal (*n* = 8)[a]	100 % (*n*=8)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	100 % (*n*=8)	0.0% (*n*=0)
P value	N.s	0.215	N.s	N.s	N.s	N.s	0.485	N.s	N.s
Group B1									
Human-CA (*n*=12)[a]	100 % (*n*=12)	33.3 % (*n*=4)	0.0 % (*n*=0)	8.3 % (*n*=1)	0.0 % (*n*=0)	0.0 % (*n*=0)	66.7 % (*n*=8)	100 % (*n*=12)	0.0 % (*n*=0)
Companion animal (*n*=4)[a]	100 % (*n*=4)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	0.0 % (*n*=0)	100 % (*n*=4)	0.0 % (*n*=0)
P value	N.s	0.516	N.s	N.s	N.s	N.s	0.077	N.s	N.s
Group B2									
Human-CA (*n*=57)[a]	100 % (*n*=57)	56.1 % (*n*=32)	28.1 % (*n*=16)	8.8 % (*n*=5)	59.6 % (*n*=34)	57.9 % (*n*=33)	98.2 % (*n*=56)	100 % (*n*=57)	0.0 % (*n*=0)
Companion animal (*n*=6)[a]	100 % (*n*=6)	83.3 % (*n*=5)	66.7 % (*n*=4)	16.7 % (*n*=1)	100 % (*n*=6)	66.7 % (*n*=4)	83.3 % (*n*=5)	100 % (*n*=6)	16.6 % (*n*=1)
P value	N.s	0.387	0.075	0.275	0.078	N.s	0.183	N.s	0.095
Group D									
Human-CA (*n*=5)[a]	100 % (*n*=5)	60.0 % (*n*=3)	20.0 % (*n*=1)	20.0 % (*n*=1)	40.0 % (*n*=2)	40.0 % (*n*=2)	100 % (*n*=5)	100 % (*n*=5)	0.0 % (*n*=0)
Companion animal (*n*=17)[a]	100 % (*n*=17)	64.7 % (*n*=11)	17.6 % (*n*=3)	0.0 % (*n*=0)	47.1 % (*n*=8)	11.8 % (*n*=2)	70.6 % (*n*=12)	100 % (*n*=17)	0.0 % (*n*=0)
P value	N.s	N.s	N.s	0.227	N.s	0.209	0.289	N.s	N.s

Legend: % percentage of strains; human community-acquired UTI; [a]The number shown is the total number of strains tested; Pap fimbriae - *pap*EF operon segment; Sfa fimbriae – *sfa*; Afa afimbrial adhesion – *afa*; alpha-hemolysin operon – *hlyA*; Cytotoxic necrotizing factor-1- *cnf*-1; aerobactin siderophore – *iucD*; *E. coli* common pilus – *ecpA;* uropathogenic specific protein- *usp*; * p value < 0.05 statistically significant; N.s. not significant to calculate.

Supplementary Table 4: Genotypic characteristics of ESBLs/AmpC -producing *Escherichia coli* strains from companion animals with UTI (N=31) from 1999-2015.

Strain	Year	Companion animal	Phylogenetic group	Clonal group	CC	Antimicrobial resistance[a]	ESBL and/or AmpC genes	Pathogenicity island markers (PAIs)	Virulence genes
FMV434/00	2000	Dog	D	ST1775	-	AMP-AMC-CTX-CAZ-FOX-SXT	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMV457/00	2000	Dog	D	ST1775	-	AMP-AMC-CTX-CAZ-FOX-SXT	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMV1953/01	2001	Dog	D	ST57	CC350	AMP-AMC-CTX-CAZ-FOX-SXT-TOB-AK	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMV203/03	2003	Dog	D	ST405	CC405	AML-AMC-CTX-CIP-NOR-ENR	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{IV536}$	*ecpA – hlyA-cnf1*
FMV5825/04	2004	Dog	B2	O25b:H4-ST131-H30Rx	CC131	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN-AK-TOB	*bla*$_{CTX-M-15}$	PAI$_{II536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-cnf1-sfaDE-papEF-iucD*
FMV6346/05	2005	Cat	B1	ST539	-	AMP-AMC-CTX-CAZ-FOX-CIP-NOR-ENR-CN	*bla*$_{CMY-2}$	none	*ecpA*
FMV3389/06	2006	Dog	D	ST354	CC354	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN-TOB	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA- hlyA-iucD*
FMV521/07	2007	Cat	B1	ST224	-	AMP-CTX-CAZ-CIP-NOR-ENR	*bla*$_{CTX-M-32}$	none	*ecpA*
FMV1630/07	2007	Dog	A	unassigned ST[b]	CC23	AMP-CTX-CAZ-SXT-CIP-NOR-ENR	*bla*$_{CTX-M-15}$	PAI$_{IV536}$	*ecpA*
FMV7261/07	2007	Dog	A	ST609	CC46	AMP-CTX-SXT-CIP-NOR-ENR	*bla*$_{CTX-M-32}$	PAI$_{IV536}$	*ecpA*
FMV635/08	2008	Cat	A	ST23	CC23	AMP-AMC-CTX-CAZ-SXT-CIP-NOR-ENR	*bla*$_{CTX-M-32}$	none	*ecpA*

FMV2777/08	2008	Cat	B2	O25b:H4-ST131-H30Rx	CC131	AMP-AMC-CTX-CAZ-CIP-NOR-ENR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-papEF-iucD*
FMV1952/10	2010	Cat	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN-TOB	*bla*$_{CTX-M-9}$-*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{IV536}$	*ecpA-papEF*
FMV25/11	2011	Cat	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF*
FMV29/11	2011	Dog	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-CIP-NOR-ENR-CN-AK	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF*
FMV469/13	2013	Dog	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN-TOB	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{IV536}$	*ecpA-papEF*
FMV1389/13	2013	Cat	D	ST648	CC648	AML-AMC-CTX-FOX-SXT-CIP-NOR-ENR-CN	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF*
FMV55/13	2013	Cat	D	ST648	CC648	AMP-AMC-CTX-FOX-SXT-ENR-NOR-CN	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-sfaDE-papEF-iucD*
FMV58/13	2013	Cat	B2	O25b:H4-ST131-H30R1	CC131	AMP-CTX-SXT-CIP-NOR-ENR	*bla*$_{CTX-M-1}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA- hlyA-sfaDE-papEF-iucD*
FMV4479/13	2013	Dog	B1	ST533	-	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-TOB	*bla*$_{CTX-M-15}$-*bla*$_{CMY-2}$	none	*ecpA*
FMV5338/13	2013	Dog	B2	O25b:H4-ST131-H30Rx	CC131	AMP-AMC-CTX-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA,cnf1-afaBC-iucD*
FMV121/14	2014	Dog	B1	ST539	-	AMP-AMC-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-1type}$	none	*ecpA*
FMV546/14	2014	Cat	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN-TOB	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF*
FMV966/14	2014	Dog	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-F-SXT-CIP-NOR-ENR	*bla*$_{CMY-2}$	II536,ICFT073,IV536	*ecpA-papEF-iucD*

FMV1549/14	2014	Cat	D	ST648	CC648	AMP-AMC-CAZ-FOX-CIP-NOR-ENR	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF*
FMV43/14	2014	Cat	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-CIP-NOR-ENR	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-cnf1-sfaDE-papEF*
FMV78/15	2015	Dog	A	ST88	CC23	AMP-CTX-CAZ-SXT-CN-TOB	*bla*$_{CTX-M-1}$	PAI$_{IV536}$	*ecpA*
FMV97/15	2015	Dog	B2	O25b:H4-ST131	CC131	AMP-AMC-FOX	*bla*$_{CMY-2}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV53}$6-PAI$_{IICFT073}$	*ecpA-usp-hlyA-cnf1-sfaDE-papEF-iucD*
FMV4995/15	2015	Dog	B2	ST372	-	AMP-CTX-CAZ	*bla*$_{CTX-M-15}$	PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-cnf1-sfaDE-papEF*
FMVCP39/15	2015	Cat	D	ST3258	-	AMP-CTX-SXT	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMV151/15	2015	Cat	D	ST648	CC648	AMP-AMC-CTX-CAZ-FOX-SXT-CIP-NOR-ENR-CN	*bla*$_{CMY-2}$	PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-hlyA-cnf1-sfaDE-papEF-iucD*

Legend: ESBLs- extended-spectrum β-lactamases; AmpC- AmpC β-lactamases; AMC- Amoxicilin/clavulanate, AMP-Ampicillin, AK- Amikacin, CAZ-Ceftazidime, CIP-Ciprofloxacin, CN- Gentamycin, CTX-Cefotaxime, ENR-Enrofloxacin, FOX-Cefoxitin, F- Nitrofurantoin, NOR- Norfloxacin, SXT- Trimethoprim/sulphamethoxazole, TOB-Tobramycin.;

[a]susceptibility was accessed according to Clinical and Laboratory Standards Institute (CLSI) guidelines M100-S29;

Pap fimbriae - papEF operon segment; Sfa fimbriae – *sfa*; Afa afimbrial adhesion – *afa*; alpha-hemolysin operon – *hlyA*; Cytotoxic necrotizing factor-1- *cnf*-1; aerobactin siderophore – *iucD*; *E. coli* common pilus – *ecpA;* uropathogenic specific protein- *usp*

[b] New ST allelic profile - Marques et al., 2017.

Supplementary Table 5: Genotypic characteristics of ESBLs/AmpC - producing *Escherichia coli* istrains from Humans-CA with UTI (N=85) from 2013.

strain	Phylogenetic group	Clonal group	CC	Antimicrobial resistance[a]	ESBL and/or AmpC genes	Pathogenicity island markers (PAIs)	Virulence genes
FMVPPL402	A	ST10	CC10	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-1}$	PAI$_{IV536}$	*ecpA-papEF*
FMVML22190	A	ST10	CC10	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-1}$	PAI$_{IV536}$	*ecpA*
FMVPCA14	A	ST88	CC23	AML-CTX-CAZ-SXT-TOB	*bla*$_{CTX-M-32}$	PAI$_{IV536}$	*ecpA*
FMVANZ1442	A	ST88	CC23	AML-AMC-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF-iucD*
FMVPLO664	A	ST90	CC23	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{IV536}$	*ecpA*
FMVANZ1364	A	ST90	CC23	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-9like}$	none	*ecpA*
FMV2222	A	ST167	CC10	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-32}$	PAI$_{IV536}$	*ecpA*
FMV1927	A	ST540	-	AML-CTX-CAZ-SXT	*bla*$_{CTX-M-32}$	none	*ecpA*
FMVPCA2118	A	ST617	CC10	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-1}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMVPRV598	A	ST5257	-	AML-CTX	*bla*$_{CTX-M-32}$	none	*ecpA*
FMVCde788	A	ST6023	-	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-14}$	none	*ecpA*
FMVML17567	B1	ND	-	AML-CTX-SXT	*bla*$_{CTX-M-2}$	none	*ecpA*
FMVMLD457	B1	ST58	CC155	AML-CTX-SXT	*bla*$_{CTX-M-1}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF-iucD*
FMVPOOK18	B1	ST58	CC155	AML-CTX-SXT	*bla*$_{CTX-M-1}$	PAI$_{IV536}$	*ecpA-papEF-afaBC*,
FMVBa417	B1	ST453	CC86	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-14}$	none	*ecpA*
FMVMQ8509	B1	ST453	CC86	AML-CTX-CAZ-CIP-NOR	*bla*$_{SHV-12}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMVJP842	B1	ST453	CC86	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-14}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF-iucD*
FMVPRV129e	B1	ST453	CC86	AML-AMC-CTX-SXT-CIP-NOR-CN	*bla*$_{CTX-M-14}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*

FMVML24561	B1	ST453	CC86	AML-CTX-CIP-NOR	*bla*$_{CTX-M-1}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMVML35760	B1	ST453	CC86	AML-AMC-CTX-CIP-NOR-CN	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMV2660	B1	ST847	-	AML-CTX-SXT	*bla*$_{CTX-M-14}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-iucD*
FMVMQ11227	B1	ST1725	-	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF-iucD*
FMVANG80	B1	ST1196	-	AML-CTX-CAZ-SXT-CIP-NOR	*bla*$_{CTX-M-1}$	none	*ecpA*
FMVMR1960	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-CAZ-SXT-CIP-NOR	*bla*$_{CTX-M-1}$	PAI$_{I536}$-PAI$_{IIJ96}$-IPAI$_{I536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVPFB 399	B2	O25b:H4-ST131-H30R1	CC131	AML-AMC-CTX-CAZ-SXT-CIP-NOR	*bla*$_{CTX-M-1}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVOM1969	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVO5N38	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVANG 39	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-afaBC-hlyA-cnf1-iucD*
FMVMLD 252	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-FOX-SXT-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{IV536}$	*ecpA-hlyA-cnf1*
FMVOMR+80	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-SXT-CIP-NOR-CN-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVNan19	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVPCV394	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*

FMVPAV806	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVNA255	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$PAI-$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVVIM 1771	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-F-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVAH6430	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF- afaBC- iucD*
FMVPME 508	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI $_{IV536}$-PAI $_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVPSF1626	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVPSF1301	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-IPAI$_{V536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVPD26e5	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-iucD*
FMVML 27331	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVOMV 168	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-CN-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
PFMVD5379	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$, PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVML25380	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*

FMVL5449	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVPD6085	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CIP-NOR-CN-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVANG82	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVJA861	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI $_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVMR1533	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVMQ11181	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVPCV690	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVLum380	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVCT313	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVML28567	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-SXT-CIP-NOR-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-afaBC-iucD*
FMVMQeeo5	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15like}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVML34063	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*

FMVAM1035	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-SXT-CIP-NOR-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-sfaDE-iucD*
FMVOSF729	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-CIP-NOR-CN-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVMQX1101	B2	O25b:H4-ST131-H30Rx	CC131	AML-AMC-CTX-CAZ-CIP-NOR-CN	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-cnf1-iucD*
FMVRR3043	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-SXT-CIP-NOR-CN-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVPcue031	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-CN-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-iucD*
FMVML36906	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-CAZ-CIP-NOR-TOB-AK	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVPME856	B2	O25b:H4-ST131-H30Rx	CC131	AML-CXT-SXT-CIP-NOR-CN-AK	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVCS4641	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$-PAI $_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVCS4642	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-afaBC-iucD*
FMVLi8696	B2	O25b:H4-ST131-H30Rx	CC131	AML-CTX-SXT-CIP-NOR-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-afaBC-iucD*
FMVAFE 711	B2	O25b:H4-ST131	CC131	AML-AMC-CTX-CAZ-CIP-NOR-CN-TOB	*bla*$_{CTX-M-15}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVPCA456	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-CAZ-CIP-NOR	*bla*$_{CTX-M-32}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVLF4809	B2	O16:H5-ST131		AML-CTX-SXT-CIP-NOR-CN-TOB	*bla*$_{CTX-M-9like}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*

FMVUF822	B2	O25b:H4-ST131-H30Rx	CC131	AML-CXT-SXT-CIP-NOR-CN	*bla*$_{CTX-M-9like}$	PAI$_{I536}$-PAI$_{IIJ96}$-$_{I}$PAI$_{I536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVML36741	B2	025b:H4-ST131	CC131	AML-AMC-CTX	*bla*$_{CTX-M-9like}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVNAL 47	B2	O25b:H4-ST131-H30R1	CC131	AML-AMC-CTX-FOX-SXT-CIP-NOR	*bla*$_{CTX-M-14}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVPF8675	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVOSJ589	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-sfaDE-hlyA-cnf1-iucD*
FMVCD3e67	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-cnf1-iucD*
FMVANZ1648	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVOSJ600	B2	O25b:H4-ST131-H30R1	CC131	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-27}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-hlyA-cnf1-iucD*
FMVRO757	B2	O25b:H4-ST131	CC131	AML-AMC-CTX-CAZ-FOX-SXT	*bla*$_{CMY-2}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVPSF1269	B2	O25b:H4-ST131	CC131	AML-AMC-CTX-FOX-F-SXT-CIP-NOR	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*
FMVRR2968	B2	O25b:H4-ST131	CC131	AML-AMC-CTX-CAZ-FOX-CIP-NOR	*bla*$_{CMY-2}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{ICFT073}$- $_{IV536}$- $_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVMQ13896	D	ST117	-	AML-CTX-CAZ-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{IIJ96}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-papEF-hlyA-cnf1-iucD*
FMVUF628	D	ST354	CC354	AML-CTX-SXT_CIP-NOR-CN	*bla*$_{CTX-M-14}$	PAI$_{ICFT073}$-PAI$_{IV536}$-PAI$_{IICFT073}$	*ecpA-iucD*

FMVMC2815	D	ST410	CC23	AML-CTX-SXT-CIP-NOR	*bla*$_{CTX-M-15}$	PAI$_{I536}$-PAI$_{IIJ96}$-PAI$_{II536}$-PAI$_{IV536}$	*ecpA-papEF-sfaDE-afaBC-hlyA-cnf1-iucD*
FMVLi3864	D	ST648	CC648	AML-AMC-CTX-CAZ-FOX-SXT-CIP-NOR	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA- iucD*
FMVPD4783	D	ST778	CC38	AML-AMC-CTX-CAZ-FOX-SXT	*bla*$_{CMY-2}$	PAI$_{ICFT073}$-PAI$_{IV536}$	*ecpA-papEF-iucD*

Legend: ESBLs- extended-spectrum β-lactamases; AmpC- AmpC β-lactamases; AMC- Amoxicilin/clavulanate, AML- Amoxicilin, AK- Amikacin, CAZ-Ceftazidime, CIP-Ciprofloxacin, CN- Gentamycin, CTX-Cefotaxime, ENR-Enrofloxacin, FOX-Cefoxitin, F- Nitrofurantoin, NOR- Norfloxacin, SXT- Trimethoprim/sulphamethoxazole, TOB-Tobramycin.; [a]susceptibility was accessed according to Clinical and Laboratory Standards Institute (CLSI) guidelines M100-S29;
Pap fimbriae - papEF operon segment; Sfa fimbriae – *sfa*; Afa afimbrial adhesion – *afa*; alpha-hemolysin operon – *hlyA*; Cytotoxic necrotizing factor-1- *cnf*-1; aerobactin siderophore – *iucD*; *E. coli* common pilus – *ecpA;* uropathogenic specific protein- *usp.*

3.2. ESBLs/ carbapenemase-producing *Escherichia coli* faecal colonization in healthy companion animals.

3.2.1. Higher ESBL/AmpC-producing Enterobacteriaceae faecal colonisation in dogs after elective surgery

Paper submmited at *Antibiotics Journal*.

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Partial results was presented as,

One Poster communication at the international congress 26th European Congress of Veterinary Internal Medicine - Companion Animals (ECVIM-CA), 2016, Goteborg, Sweeden

Work deserved the award of “Best poster presentation” at the 26th European Congress of Veterinary Internal Medicine - Companion Animals (ECVIM-CA), given by the International Society for Companion Animal Infectious Diseases (ISCAID).

Higher ESBL/AmpC-producing Enterobacteriaceae faecal colonisation in dogs after elective surgery

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Abstract

The purpose of this study was to evaluate the presence and load of ESBL/AmpC-producing Enterobacteriaceae faecal carriage in healthy dogs and to identify potential risks factors associated with faecal colonization. Faecal samples were collected from dogs submitted to surgical procedures (*n*=25). Faecal samples from the surgery group were collected before surgery (BS) and after surgery (AS). β-lactamases were detected by PCR. Statistical analyses were performed with SAS software (v.9.4), a *p* value ≤ 0.05 was considered statistically significant. ESBL/AmpC-producing Enterobacteriaceae bacteria implicated in this study were *E. coli*, *K. pneumoniae* and *E. cloacae*. TEM and CTX-M-1 group genes were the most frequent β-lactamases detected. The number of dogs colonized with 3GC-resistant Enterobacteriaceae bacteria was significantly higher in AS (63.6%, *n*=14/22) comparing to BS (20.0%, *n*=5/25, *p*= 0.0033). The ESBL/AmpC-producing bacteria load was significantly higher in the AS group when compared with BS (*p*=0.025). This study shows that 3GC-resistant Enterobacteriaceae and ESBLs/AmpC producers in veterinary clinical practice is a concern and highlights the need to implement preventive measures to minimize its spread.

Keywords: Veterinary Hospitals; Antimicrobial prophylactic use; ESBLs; pAmpC; Third generation cephalosporin resistance; Gut colonization.

Introduction

The European Medicine Agency has reviewed the public health risks associated with the transfer of antimicrobial resistance from companion animals and has identified the major microbiological hazards coming from companion animals to humans, including third-generation cephalosporin resistant bacteria [1]. The presence of antimicrobial resistant bacteria in companion animals and their close contact with humans provides opportunities for interspecies transmission [2]. In veterinary hospitals, infections acquired during hospitalization caused by resistant bacteria are an increasing problem [3–5]. Antimicrobials are regularly used for the prevention and control of infections in companion animals, and many of the antimicrobials used are the same or closely related to those used in the treatment of bacterial infections in humans [6,7].

β-lactams are among the most important antimicrobials used in veterinary medicine. β-lactam resistance can be attained through intrinsic and/or acquired antimicrobial resistance. Intrinsic resistance mechanisms are those specified by naturally occurring genes found on the hosts' chromosome, such as, AmpC β-lactamase [8]. Acquired resistance genes, such as most β-lactamases, may enable bacteria to destroy the antimicrobial agent, leading to clinical resistance. These mechanisms cause increased antimicrobial resistance through different pathways: modification of the antimicrobial target site; expression of efflux systems that prevent the antimicrobial from reaching its intracellular target; and acquisition of mutations that limit the access of the antimicrobial agents to the intracellular target site via downregulation of porin genes [9]. Extended-spectrum β-lactamases (ESBLs) were first reported by Knothe et al. [10] as transmissible resistance determinants to newer types of cephalosporins which, thereafter, have emerged as a major source of antimicrobial resistance in gram-negative pathogens [10].

ESBLs and plasmid-mediated cephamycinases (*p*AmpC), involve mutations in genes targeted by the antimicrobial and the transfer of resistance determinants borne on plasmids, and other mobile genetic elements carrying resistance genes, such as plasmid-encoding β-lactamases and transposons can easily be transmitted by conjugation to other bacteria, even across species. ESBL producers are mostly *Escherichia coli* and *Klebsiella pneumoniae* and are the main source of community- and hospital-acquired infections in human and veterinary medicine [8,11–13].

Prophylactic antimicrobial use involves the administration of the antimicrobial in the absence of infection, with the aim of preventing it, for example in the perioperative period [7]. Ideally, the antimicrobial prophylaxis scheme should be selected and prescribed in order to minimize the possible impact on the normal bacterial flora of the patient and on the microbiologic ecology of the hospital [14]. Inappropriate prophylaxis may promote the selection of antimicrobial-resistant isolates [7].

The gastrointestinal tract is one of the main reservoirs for the emergence and dissemination of antimicrobial-resistant bacteria. Dog faeces are a recognized source of relevant resistant bacteria that can be transmitted to humans through direct contact or through shared (domestic and public) environments [2,15–21]. Antimicrobial resistance to third generation cephalosporins (3GC) has been already detected in bacteria from canine faecal samples in the last years [19,20,22,23]. However, to the best of our knowledge this is the first study to understand the dynamics of ESBLs/AmpC producing- Enterobacteriaceae in intestinal tract of healthy dogs that went to the Veterinary Teaching Hospital from the Faculty of Veterinary Medicine - University of Lisbon to perform elective surgical procedures. The purpose of this study was to evaluate the presence and load of ESBLs/AmpC-producing Enterobacteriaceae faecal carriage in healthy dogs undergoing surgery and to identify potential risks factors associated with faecal colonization.

Results and Discussion

Of the faecal samples obtained from healthy dogs 36.0 % (*n*=9/25) were females and 64.0 % (n=16/25) were males, with a median age of 7 years (ranging from 0.2 - 13 years). All the animals were from a private owner. Previous hospitalization in last year was observed in 60.0 % (*n*=15/25) of the dogs and 50.0 % (*n*=12/24) had been treated with an antimicrobial agent within the last year. All the animals had street access, 68.0 % (*n*=17/25) and 12.0 % (n=3/25) of the dogs had cohabitation with other animals and shelter/hotel access, respectively.

About 76.0 % (*n*=19/25) of the animals were submitted to soft tissues surgery and 24.0 % (*n*=6/25) to orthopaedic surgery. Regarding prophylactic antimicrobial treatment 92.0 % (*n*=23/25) of the dogs received prophylactic antimicrobial through oral administration by different antimicrobials before and after surgery. About 52.0 % (*n*=12/23) of the dogs and 73.9 % (*n*=17/23) were administered AMC, before surgery and after surgery, respectively. One dog received AMC and a second-generation cephalosporin (2GC) after surgery; three dogs received other antimicrobials (2GC, *n*=1; AMC and metronidazole (MET), *n*=1; and MET and macrolides *n*=1) in both time points.

In this study, 20.0 % (*n*=5/25) of the dogs on admission to the hospital (BS) were colonized with ESBL-producing Enterobacteriaceae. Most of the colonized dogs harboured ESBL producing- Enterobacteriaceae *E. coli* (*n*=4) belonging to the commensal phylogenetic group-B1 (*n*=3) (Table 1). The most common antimicrobial resistance phenotype of BS *E. coli* isolates was AMP^R–AMC^R-KF^R-CTX^R-FOX^R-CAZ^R (60.0 %, n=3/4). Two *E. coli* harboured the bla_{TEM} gene, one harboured bla_{SHV} gene and one $bla_{CTX\text{-}M\text{-}1group}$ (Table 1).

Regarding samples collected after surgery (AS), about 64.0 % (*n*=14/22) of dogs were colonized with 3GC-resistant Enterobacteriaceae. Around 45.0 % (*n*=10/22) of the faecal

samples were *E. coli* positive, followed by *K. pneumoniae* (18.2%, *n*=4/22) and *E. cloacae* (13.6%, *n*=3/22) (Table 2).

Among *E. coli* isolates, the most common antimicrobial resistance phenotype was AMP^R-AMC^R-KF^R-CTX^R-FOX^R-CAZ^R (*n*=8/10), while among *K. pneumonia* and *E. cloacae* the most common was AMP^R-AMC^R-KF^R-CTX^R-CAZ^R (*n*=2/4) and AMP^R- KF^R- CTX^R- FOX^R- CAZ^R (*n*=3/3), respectively. Furthermore, 70.0 % of the *E. coli* isolates (*n*=7/10) harboured the bla_{TEM} gene, and the remaining isolates harboured $bla_{CTX\text{-}M\text{-}1group}$ gene (*n*=1/10), bla_{SHV} gene (*n*=1/10) and one carried the combination of $bla_{OXA\text{-}1}$+bla_{TEM} genes (Table 2). Regarding *K. pneumoniae,* all isolates were positive for $bla_{OXA\text{-}1}$+bla_{TEM}+$bla_{CTX\text{-}M\text{-}1group}$ genes (Table 1). Besides the increase in 3GC-resistant Enterobacteriaceae in faecal samples after surgery (AS), most *E. coli* isolates belonged to commensal phylogenetic groups (group-A, *n*=4/10; group-B1, *n*=3/10). Pathogenic phylogenetic groups were also detected (group-B2, *n*=2/10; group-D, *n*=1/10) (Table 1). However, there has no statistical significant difference between BS and AS regarding pathogenic *E. coli* phylogenetic groups.

In this study the number of dogs colonized with ESBL/AmpC-producing Enterobacteriaceae was significantly higher in AS (63.6%, *n*=14/22) comparing to BS (20.0%, *n*=5/25, *p*= 0.0033) (Table 1). Moreover, the ESBL/AmpC-producing Enterobacteriaceae load mean in AS group was $1.74x10^6 \pm 5.33x10^6$ CFU/g, and in BS group it was $1.10x10^2 \pm 4.51x10^2$ CFU/g. The CTX-resistant bacteria faecal load was statistically significantly higher in the AS group when compared with BS (*p*=0.025) (Table 2).

Statistical analysis was performed to determine possible risk factors associated with the faecal carriage of ESBL/AmpC-producing Enterobacteriaceae in healthy dogs. After applying the backwards elimination procedure in logistic regression none of the variables were statistically significant.

In this study, about 20.0% of dogs before surgery and before entering in the hospital were already colonized with ESBL/AmpC-producing Enterobacteriaceae. The results obtained here were similar to those previously published using samples from 2010-2011 from healthy dogs [19]. Likely, this similarity is related with the fact that both studies were conducted in the same geographical area (metropolitan region of Lisbon). A significant increase in antimicrobial resistance was detected among bacteria causing UTI in companion animals from the Lisbon area between 1999 and 2014 [25]. Therefore, the apparent stable frequency of the CTX-resistant bacteria faecal carriage among heathy dogs is considered a positive outcome from this study. Nevertheless, the frequency of colonization by CTX-resistant bacteria here reported (20%) before surgery should not be neglected since faecal carriage of ESBLs/AmpC-producing may be a risk factor for secondary infections by MDR bacteria in hospitalized patients, as it occurs in humans.

The ESBL/AmpC-producing Enterobacteriaceae detected in this study were *E. coli*, *K. pneumoniae* and *E. cloacae*. *Enterobacter cloacae* is ubiquitous in the environment [26] and it is

commensal in the intestinal tract of humans and animals. This species is also prone to contaminate various medical, intravenous, and other hospital devices contributing to skin/soft tissue infections, urinary tract and intra-abdominal infections and others [26]. In *E. cloacae* isolates from this study, the phenotype of resistance to cefotaxime and ceftazidime could not be explained by the presence of ESBL- and/or AmpC β-lactamase genes, indicating that other mechanisms of resistance could be present, such as, bla_{GES} and bla_{VEB} genes or other AmpC β-lactamases [27]. Furthermore, *E. cloacae* has an intrinsic resistance to ampicillin, amoxicillin, first-generation cephalosporins, and cefoxitin owing to the production of constitutive AmpC β-lactamase. Resistance of *Enterobacter* spp. to 3GC is in most of the cases caused by overproduction of AmpC β-lactamases [26,28].

In this study, the bla_{TEM} and $bla_{CTX\text{-}M\text{-}1group}$ genes were the most frequent β-lactam-resistance genes, which is in agreement with previous studies [19,29].

Hordijk et al. [22] analysed healthy dogs and cats without contact with the hospital environment in the Netherlands and detected a high percentage (45%) of dogs colonized with Enterobacteriaceae producing β-lactamases (ESBL/AmpCs). Procter et al. [30] reported that 12.7% of *E. coli* strains isolated from dogs, who attended parks in three cities in Canada, were resistant to β-lactam antimicrobials. Aslantas et al. [31] detected 22 % of dogs were colonized by CTX-resistant *E. coli* in Turkey. The different frequencies of β-lactam resistant bacteria detected in these studies may be related to differences among geographical regions or to differences between study designs. Nevertheless, it highlights the importance of reporting data from different geographical regions.

In this study, ESBL-producing Enterobacteriaceae significantly increased during antimicrobial administration and changes in fecal microbiota occurred, which could be in part explained by the prophylatic use of amoxicillin-clavulanate. The use of β-lactams has been previously associated with an increased risk of carriage of antimicrobial resistant *E. coli* in dogs [19,32,33]. However, in this study, antimicrobial treatment within the last year was not identified as a potential risk factor, unlike what has been previously described [19,32]. The small sample size used in this study is considered a limitation that could have hampered the detection of additional risk factors. Nevertheless, the findings here presented regarding dog colonization by CTX-resistant bacteria are of public-health and veterinary interest.

In one recent study conducted in the Netherlands using whole genome sequencing [34], around 43% of owned dogs were found to be persistently colonized by ESBL-producing Enterobacteriaceae (6 months). It is important to notice that van den Bunt et al. used pre-enrichment media unlike the study here presented. Therefore, the high frequency of colonized dogs by CTX-resistant bacteria detected after surgery (64.0 %) could be even higher.

An important finding from this study is not only that the number of colonized dogs by CTX-resistant bacteria increased significantly during antimicrobial treatment, but also that there was

an significant increase in the detected faecal load (UFC/g), achieving a mean value of 1.74×10^6 UFC/g. These two findings together further highlight the importance of dogs in the dissemination of resistant bacteria and emphasize the need for appropriate faecal disposal during antimicrobial prophylaxis or treatment. Future longitudinal studies should be conducted to access the evolution of the faecal CTX-resistant bacteria load over time once the antimicrobial treatment in interrupted.

It was also interesting to notice, that a higher diversity of CTX-resistant bacteria was found after surgery, including bacterial species frequently associated with nosocomial infections, namely *K. pneumoniae* and *E. cloacae.* This finding is of great importance not only because of the direct impact on patients, but also because resistant bacteria can be transmitted from companion animals to humans and disseminated into the environment [2,15,17,18].

ESBL/AmpC-producing Enterobacteriaceae may also spread from patient-to-patient due to inadequate attention to infection control measures, especially hand washing. Infections caused by Enterobacteriaceae have features that are of particular concern. These organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antimicrobial drug resistance, especially in the presence of antimicrobial selection pressure [35].

Nowadays, there is evidence that the composition of the gut microbiota may change in response to external factors such as antimicrobials and environment [36]. Antimicrobials administration creates alterations in the faecal microbiome and can affect the immune system and the health of the host [37,38].

Antimicrobials must be used responsibly and restrictively to minimize resistance, retain the efficacy of the currently available antimicrobial agents, and to maintain a healthy gut microbiome [36]. However, more studies are needed to understand what happens on the gut flora after cessation of the antimicrobial treatment and in the following months.

Materials and methods

Sampling procedure and collection of data

From February to July 2014, faecal samples were obtained from 25 healthy dogs (without signs of gastrointestinal disease in the previous week) that went to the Veterinary Teaching Hospital from the Faculty of Veterinary Medicine - University of Lisbon to undergo a surgical treatment. The surgery study group was divided in: i) before surgery (BS) - upon admission to the Veterinary Hospital and ii) after the surgical procedure (AS). Animals were excluded if they had been treated with an antimicrobial agent in the previous month. The surgical procedures considered for this study were soft tissue (e.g orchiectomy, ovariohysterectomy) and orthopaedic surgery (e.g., ball joint dislocation and hemilaminectomy). Faecal samples were collected at two different time points, namely before (BS) and after surgery (AS). A total of 25 animals were included in the BS group. The follow up samples included in the AS group were collected one

week after surgery. However, in three animals the follow- up was not possible and, therefore, 22 samples were studied.

Data about potential risk factors for ESBL/AmpC-producing Enterobacteriaceae faecal carriage was obtained through a questionnaire to the owner regarding: age, gender, hospitalisation and antimicrobial treatment within the last year, cohabitation with other animals, street access, shelter/hotel access, and surgery type (soft tissue, orthopaedic) and surgery reason (elective surgery or non-elective). To avoid categories with very unbalanced numbers, samples/information obtained from surgery type were combined by soft tissues and orthopaedic surgery.

The dog owners were questioned verbally, and all replied to the questions listed above. The faecal sample collection was conducted by the owners using non-invasive methods. Owners were given specific instructions about the collection method to avoid sample contamination through contact with the ground. These also included the faecal collection into sterile containers and the use of gloves.

Bacteria isolation, identification and DNA extraction

Faecal samples were directly transported to the Laboratory of Antibiotic Resistance, FMV-UL, Lisbon, Portugal. In total, 1 g of faeces was diluted in sterile saline solution (NaCl, 0.85%-Merck - Germany). Once homogenized, 10 µL were directly cultured on MacConkey agar plates (Scharlau, Spain) supplemented with 2.0 µg/ml of cefotaxime (CTX) (Sigma-Aldrich, USA) and incubated overnight at 37 °C. CTX-resistant Enterobacteriaceae bacteria were then quantified by counting the colony-forming units (CFU) per gram of faeces. Positive samples were screened for the presence of different colony morphologies of CTX-resistant Enterobacteriaceae. One isolate of each unique morphology was selected and further studied from all positive faecal samples.

The bacterial species were determined using API 20E kit, the software APIWEB (Biomérieux, France) and by species-specific PCR [40,41]. DNA extraction was conducted using a boiling method [42].

***Escherichia coli* Phylogenetic typing**

Phylogenetic typing was performed in all E. coli isolates to determine the main phylogenetic groups (A, B1, B2 and D) according to the amplification of chuA and yjaA genes, and TspE4C2 fragment) by multiplex PCR [43].

Antimicrobial Susceptibility testing

Antimicrobial susceptibility testing and interpretation were performed using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI 2018). The following antimicrobial disks (Oxoid, Hampshire, UK) were used: 10 µg ampicillin

(AMP), 30 µg amoxicillin-clavulanate (AMC), 30 µg cefalotin (KF), 30 µg cefotaxime (CTX), 30 µg ceftazidime (CAZ) and 30 µg cefoxitin (FOX). The reference strain *E. coli* ATCC 25922 was used for quality control testing. ESBL-production was confirmed by the double-disk synergy test according to CLSI standards and isolates were classified as susceptible or resistant according to CLSI criteria [44].

ß-lactamases resistance genes

3GC-resistant Enterobacteriacea isolates were screened by PCR for the presence of *bla*$_{SHV}$, *bla*$_{OXA-1}$, *bla*$_{TEM}$ β-lactamase genes; *bla*$_{CTX-M}$, *bla*$_{CTX-M-1group}$ and *bla*$_{CTX-M-9group}$ ESBLs [42–44]; and *bla*$_{MOX-1}$, *bla*$_{MOX-2}$, *bla*$_{CMY-1}$, *bla*$_{CMY-8}$ *to bla*$_{CMY-11}$, *bla*$_{LAT-1}$ *to bla*$_{LAT-4}$, *bla*$_{CMY-2}$ *to bla*$_{CMY-7}$, *bla*$_{BIL-1}$, *bla*$_{DHA-1}$, *bla*$_{DHA-2}$, *bla*$_{ACC}$, *bla*$_{MIR-1T}$, *bla*$_{ACT-1}$ and *bla*$_{FOX-1}$ *to* $_{FOX-5}$*b p*AmpC encoding genes [48]. Negative and positive controls were used for all PCRs.

Statistical Analysis

Statistical analysis was performed using SAS statistical software package for Windows, version 9.4 (SAS Institute, Cary, NC). For the categorical variables, proportions were compared using Fisher's exact test and General linear model (GLM) procedures were used to performed descriptive statistics of ESBL/AmpC-producing Enterobacteriaceae load. The results were considered statistically significant when $p < 0.05$.

Independent (risk factor) variables were derived from information from the owner questionnaire. Six potential independent variables were categorical. The outcome for each sample was the presence or absence of ESBL/AmpC-producing Enterobacteriaceae. Each response variable was analysed independently by Logistic regression with a final model constructed by backwards elimination. The final model for each response variable retained only the factors where the Wald $\chi 2$ test had $P<0.10$ [49].

Conclusions

The findings of the current research showed that about 20% of dogs before surgery and before entering in the hospital were already colonized with ESBL/AmpC-producing Enterobacteriaceae, mainly harbouring the *bla*TEM and blaCTX-M-1group genes. After elective surgery the number of dogs colonized with ESBL/AmpC-producing Enterobacteriacea and the mean load of ESBL/AmpC-producing Enterobacteriaceae was significantly higher than before surgery. In this study, antimicrobial treatment within the last year was not identified as a potential risk factor. Yet, ESBL-producing Enterobacteriaceae significantly increased during antimicrobial prophylatic use and changes in fecal microbiota occurred. European and National appropriate antimicrobial surgical prophylaxis guidelines are urgently needed for the compliance of antimicrobial stewardship principles in veterinary hospitals.

Author Contributions

Conceptualization, J.C., A.B. and C.P.; methodology, A.B. and J.C.; validation, C.M. and C.P.; formal analysis, A.B. and L.T.D.G.; investigation, A.B., C.M. and J.C.; data curation, A.B. and J.C.; writing—original draft preparation, A.B. and J.C.; writing—review and editing, C.P. and L.T.D.G.; supervision, C.P.; project administration, A.B.; funding acquisition, C.P. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest statement

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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Table 1: Genotypic and phenotypic traits of ESBL/AmpC-producing Enterobacteriaceae from healthy dogs in the surgery group, on admission to veterinary hospital-before surgery (BS) and after surgery (AS).

Animal group	ESBLs (%)	*p* value	Isolates ID	Bacteria	Antimicrobial resistance phenotype	β-lactamases	*E. coli* phylogroup
Surgery group on admission to Hospital (BS) (*n*=25)	20.0		FMVS1	*E. coli*	AMP KF CTX	*bla*$_{\text{CTX-M-1group}}$	B1
			FMVS2	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	D
			FMVS14	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{SHV}}$	B1
			FMVS18	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	B1
			FMVS20	*K. pneumoniae**	AMP AMC KF CTX FOX	*bla*$_{\text{SHV}}$	-
Surgery group after surgery (AS) (*n*=22)	63.6	0.0033	FMVS1	*K. pneumoniae**	AMP AMC KF CTX CAZ	*bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1group}}$,	-
			FMVS2	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	D
			FMVS3a	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	B2
			FMVS3b	*K. pneumoniae**	AMP AMC KF CTX CAZ	*bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1group}}$	-
			FMVS3c	*K. pneumoniae**	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$, *bla*$_{\text{CTX-M-1group}}$	-
			FMVS3d	*K. pneumoniae**	AMP AMC KF CTX	*bla*$_{\text{OXA-1}}$, *bla*$_{\text{CTX-M-1group}}$	-
			FMVS4	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS6	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS7	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	B1
			FMVS9	*E. coli*	AMP KF CTX	*bla*$_{\text{CTX-M-1group}}$	B2
			FMVS11	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{OXA-1}}$, *bla*$_{\text{TEM}}$	A
			FMVS12	*E. cloacae* **	AMP KF CTX FOX CAZ	nd	-
			FMVS13	*E. coli*	AMP KF CTX CAZ	*bla*$_{\text{SHV}}$	B1
			FMVS16	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	A
			FMVS17	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	A
			FMVS21	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	B1
			FMVS25	*E. coli*	AMP AMC KF CTX FOX CAZ	*bla*$_{\text{TEM}}$	A

Legend: AMP-Ampicillin; AMC-Amoxicillin/clavulanic acid; CTX-Cefotaxime; CAZ-Ceftazidime; FOX-Cefoxitin; KF-Cephalotin; nd-not detected. **K. pneumoniae* is intrinsic resistant to AMP; ** E. cloacae is intrinsic resistant to AMP, AMC, first-generation cephalosporins, and FOX [24].

Table 2: Descriptive statistics of ESBL/AmpC-producing Enterobacteriaceae load (CFU/g of faeces) per dog in the before surgery group (n=25) and after surgery group (n=22) by the General linear model procedure.

Animal group	Mean (CFU/g)	SD	SE	Min (UFC/g)	Max (UFC/g)	*p* value
Before surgery group (*n*=25)	$1.10x10^{2\ a}$	$4.51x10^{2}$	$5.24x10^{5}$	0.0	$2.25x10^{3}$	0.025
After surgery group (*n*=22)	$1.74x10^{6\ b}$	$5.33x10^{6}$	$4.00x10^{5}$	0.0	$1.84x10^{7}$	

Legend: SD-Standard deviation; SE- Standard error mean; Min-minimum; Max-maximum. Mean values with a character with different letters are statistically significant (*p* value = 0.025)

Chapter 4

ESBLs/ carbapenemase - producing Extraintestinal pathogenic *Escherichia coli* in companion animals and humans with direct contact

4.1. Characterization of the gut colonization by *Escherichia coli* and the resistance genes and virulence between companion animals and humans living in close contact.

4.1.1- Sharing of clinically important antimicrobial resistance genes by companion animals and their human household members.

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Sharing of clinically important antimicrobial resistance genes by companion animals and their human household members.

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Running title: Sharing of antimicrobial resistance genes

Abstract

The aims of this study were to implemented a rapid easy methodology, to characterize the antimicrobial resistant gene gut content associated with *Enterobacteriales* and staphylococci; and to evaluate statistical association between antimicrobial resistance genes present in fecal samples from healthy companion animals and their human household members.

Fecal samples were collected from 27 humans and 29 companion animals living in close contact in 20 households. Nineteen healthy humans without daily contact with companion animals were the control group. After DNA extraction, β-lactamases families and 10 genes of other antimicrobials classes were screened by PCR. Furthermore, third-generation cephalosporin (3GC)-resistant, carbapenem-resistant and colistin -resistant *Enterobacteriales* and methicillin resistant staphylococci (MRS) were screened by bacteriological methods.

The $bla_{\text{TEM-1B}}$ gene with a P3 promotor was the most frequent β-lactam-resistant gene detected in humans and companion animals from households (33.3%, and 17.2 %, respectively). *sul2* was the most frequently shared gene by humans and animals from the same household. In fifty percent of households at least one antimicrobial resistance gene was detected simultaneously in companion animal/owner pairs. Healthy humans and companion animals carried several antimicrobial resistance genes of clinical importance. To the best our knowledge, this study reports the first detection of the $bla_{\text{SHV-27}}$ gene in fecal samples from healthy humans in Portugal and in Europe.

Keywords: Enzymes Genes, *Enterobacteriales*, staphylococci, Public health, Antibiotics.

Introduction

The spread of antimicrobial resistance is one of the greatest challenges faced nowadays in human and veterinary medicine. Antimicrobial-resistant bacterial pathogens are widespread in humans, animals and the environment, and antimicrobial-resistant infections in humans account for substantial morbidity and mortality, alongside with staggering economic costs.[1] During the last fifty years, the number of companion animals has substantially increased to the point that in many regions, the majority of people have regular and intensive contact with pets.[2]

β-lactams and potentiated sulphonamides are in many cases the first-line empirical antimicrobial therapeutic of choice either in human or veterinary medicine and therefore resistance leads to important therapeutic failures.[1] It is known that the use of antimicrobials in therapeutics and agriculture increases the selection of antimicrobial resistance and the risk of the gut colonization by antimicrobial-resistant bacteria.[3,4] The close contact of companion animals with humans provides excellent opportunities for interspecies transmission of resistant bacteria and their resistance genes, in either direction.

The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals were reviewed in the European Medicine Agency and in its Antimicrobial Working Party reflection paper, warning on the existence of antimicrobial resistance microbiological hazards coming from companion animals to humans.[1,5] There is a gap of knowledge on the dynamics of transmission and selection of antimicrobial resistance genes at the companion animal-human interface. Animals may exchange antimicrobial-resistant bacteria and resistance genes with humans, but the extent to which this happens is unknown. Yet, this information is critical to establish the measures to be implement in order to decrease the spread of antimicrobial resistance genes.

The objectives of this study were implemented a rapid easy methodology, to characterize the antimicrobial resistant gene gut content associated with *Enterobacteriales* and staphylococci in healthy companion animals and their human household members and identify which antimicrobial resistance genes were shared by both; and to evaluate statistical association between antimicrobial resistance genes present in fecal samples. The antimicrobial resistance genes studied were responsible for phenotypic resistance to six antimicrobial classes: β-lactams, aminoglycosides, colistin, trimethoprim/sulfamethoxazole, tetracycline and chloramphenicol in *Enterobacteriales* and in *Staphylococcus* spp.

Material and Methods

Sampling and collection of data

During April 2016 to November 2016, fecal samples were obtained from households (*n*=20) constituted of healthy humans (*n*=27) living with healthy companion animals (*n*=29, 9 cats and 20 dogs). A control group composed of 19 healthy humans without daily contact with

companion animals were also enrolled. Humans and companion animals were not eligible for this study if they had been under antimicrobial treatment or were hospitalized in the previous month; and if they suffered from vomiting or diarrheal disease in the last 3 months prior to the study. Ethical approval for this study was obtained from the Comissão de Ética e Bem-Estar Animal (CEBEA) from the Faculty of Veterinary Medicine of the University of Lisbon. Informed consent was obtained from all human participants. The pet owners were informed of the procedures and conducted the fecal sample collection from their companion animals with sterile containers, gloves, plastic bags or for humans themselves with the option of using Faeces collection paper Fe-Col® (Alpha Laboratories Ltd, United Kingdom) that was then transferred to a sterile plastic bag. Humans and companion animals were sampled using non-invasive methods.

A brief epidemiological questionnaire about each animal was filled by the owner containing information about age, gender, origin, contact with hospital environment, antimicrobial use in the last year, hospitalization in the last year, kennel/hotel access and lifestyle (indoor or outdoor). Also, a brief epidemiological questionnaire about each human was filled with age, gender, if employee or student in human or veterinary healthcare, prior antimicrobial treatment or hospitalization within the last year. In order to maintain the anonymity, a code number was given to the questionnaires and to samples. To ensure that inclusion was anonymous, humans control group, households, humans and animals were coded with numbered letters HC, S, H and A, respectively.

Immediately after collection, fecal samples were stored at 4°C until processing and were aliquoted and preserved at −80°C until genomic DNA extraction.

Genomic DNA extraction and purification

Fecal samples were homogenized with appropriate aseptic techniques and avoiding aerosolizing. Undigested food fragments were removed. Genomic DNA extraction was conducted using an NZY Tissue gDNA isolation commercial Kit (NZYtech-Genes & Enzymes, Portugal) with some modifications to the manufacturer's instructions. Briefly, approximately 250 mg of feces were added to 1 ml of TE buffer (10 mM tris/HCl; 1mM EDTA, pH=8). Samples were mixed by vortex and centrifuged during 15 minutes at 4000g. The supernatant was removed, and the pellet was suspended in 0.5 ml of buffer NT1. To 200 µl of the suspended sample 180 µl of buffer NT1 and 25 µl of Proteinase K solution were added. The samples were then incubated overnight at 65ºC followed by 10 minutes at 95ºC. The remaining steps were done according to the manufacturer´s recommendations.

PCR amplification for genomic DNA from fecal samples

All PCR reaction mix was prepared to a final volume of 50 µl containing nuclease-free water, DreamTaq 10X Buffer (according to the manufacturer's instructions), 0.0 to 1 µM of 25 mM $MgCl_2$, 0,2 mM of 25 mM deoxyribonucleotide triphosphates (dNTPs), 1 to 2 µM of each forward and reverse primer, 10 µg per reaction of bovine serum albumin (BSA) (Sigma-Aldrich,St. Louis, USA), 2U Dream*Taq* (5U/µL) (Fermentas ThermoScientific, Chicago, USA) and 5 µl of genomic DNA.

Negative and previously sequenced positive controls were included in all PCR reactions for quality control. PCR amplification of the 16S ribosomal DNA gene was conducted in all samples as a quality control for genomic DNA quality.[6] Oligonucleotides used in this study can be found in supplementary Table S1.

Detection of antimicrobial resistance genes (AMR)

Regarding *Enterobacteriales* five serine-β-lactamases molecular class A families of genes were screened by PCR: *bla*$_{SHV}$ and *bla*$_{TEM}$ genes and the *bla*$_{CTX-M}$ genes belonging to the *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-9}$ groups and positive amplicons were purified and submitted to nucleotide sequencing.[7-11]

The genes encoding the serine-β-lactamases molecular class C family of AmpC β-lactamases *bla*$_{MOX-1}$, $_{MOX-2}$, $_{CMY-1}$, $_{CMY-8}$ to $_{CMY-11}$, *bla*$_{LAT-1}$ to $_{LAT-4}$, $_{CMY-2}$ to $_{CMY-7}$ and $_{BIL-1}$, *bla*$_{DHA-1}$, $_{DHA-2}$, *bla*$_{ACC}$, *bla*$_{MIR-1T}$, $_{ACT-1}$, *bla*$_{FOX-1}$ to $_{FOX-5b}$ were screened by multiplex PCR.[12] Positive samples for the group *bla*$_{LAT-1}$ to $_{LAT-4}$, $_{CMY-2}$ to $_{CMY-7}$ and $_{BIL-1}$ and *bla*$_{DHA}$ were submitted to amplification of the *bla*$_{CMY-2}$ and *bla*$_{DHA-1}$ genes and submitted to nucleotide sequencing.[4] Furthermore, the genes encoding the serine-β-lactamases molecular class D family of carbapenemases *bla*$_{OXA}$, *bla*$_{BIC}$, *bla*$_{NDM}$, *bla*$_{KPC}$, *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{SPM}$ also were screened by multiplex PCR. [13]

Sulfonamide (*sul1*, *sul2* and *sul3*), trimethoprim (*dfrIa [*targeting *dfrA1, dfrA5, dfrA15, dfrA15b, dfrA16, dfrA16b])*, tetracycline (*tet*(A)), chloramphenicol (*cmlA*) and aminoglycoside (*aac(6')-Ib*) resistance genes were also screened by PCR.[14-16-]Positive amplicons of *aac(6')-Ib* gene were purified and submitted to nucleotide sequencing.

Moreover, the presence of five colistin plasmid-mediated resistance genes (*mcr*-1 to *mcr*-5) were screened by multiplex PCR.[17,18]

Regarding staphylococcal were screened by PCR the following resistance genes: to beta-lactams (*blaZ* and *mec*A) [6], to trimethoprim [*dfr*(G) and *dfr*(K)], to tetracyclines [*tet*(M) and *tet*(K)] and to chloramphenicol (*cat*$_{pC221}$). [19,20]

DNA purification and sequencing

The PCR products were purified using the NZYTech Gel Pure Kit (NZYtech-Genes & Enzymes, Portugal), according to the manufacturer's protocol, and sequencing was performed by a commercial laboratory (Stabvida, Portugal). Sequences were analyzed using the Mega (v.7) software (http://www.megasoftware.net) and Nucleotide database and Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov).

Bacteriological methods

One gram of homogenized fecal sample was added to 10 ml of sterile 0.85% NaCl (Merck, Germany) solution and mixed thoroughly. Ten microliters of fecal suspension were plated onto MacConkey (MCK) agar plates (Scharlau, Spain), with 1.5 µg/ml of cefotaxime (CTX) (Sigma-Aldrich, USA) or meropenem (MEM) (Sigma-Aldrich, USA) supplementation for detection of ESBLs or AmpC and carbapenemases – producing *Enterobacteriales.* Moreover, SuperPolymyxin medium plates was used to screening colistin plasmid-resistant *Enterobacteriales*. [21]

Furthermore, Brilliance™ MRSA plates (Oxoid, Spain) were used to detect methicillin-resistant *Staphylococcus* spp. To improve detection of low numbers of *Enterobacteriales* and methicillin-resistant *Staphylococcus* spp, 1 g of feces was added to 5 ml of sterile buffered peptone water (Biokar Diagnostics, France), vortexed, and incubated at 36 ± 1°C for 18 h. A negative quality control consisting of buffered peptone water alone was also incubated. Following incubation, 1 µl of buffered peptone water fecal suspension was plated onto the MacConkey agar, SuperPolymyxin medium and Brilliance™ MRSA plates described above. Plates were incubated at 36 ± 1°C for 18 h. Furthermore, suspected colonies obtained were quantified and to have presumptive identification, were streaked onto UriSelect agar plates (Bio-Rad, USA). All fecal samples had a high number of CFU (colony-forming unit) after direct plating onto Columbia+ 5 % blood sheep (Biomériux, France) and MacConkey agar plates, thus confirming fecal sample viability.

Statistical analysis

Statistical analysis was performed on SAS statistical software package for Windows, version 9.4, (SAS Institute Inc, Cary, North Carolina, USA). Fisher's exact test was used with a significant p value of ≤ 0.05.

Results

Enrollment and questionnaire analyses

Among the 20 households included, the household composition varied in the number of humans and companion animals, twelve households had one companion animal and one human, two households had more than one human and only one companion animal, three households had one human and more than one animal and three households had multiple humans and companion animals. Companion animal owners (*n*=27) presented ages from 8 to 66 years-old, 20 were females and 7 were males. Fifty-two percent (*n*=14/27) were employees or students in human or veterinary healthcare institutions, 33.3 % (*n*=9/27) had antimicrobial treatment during the last year and 11.1 % (*n*=3/27) were hospitalized in the previous year. Regarding the human control group (*n*=19), the ages ranged between 22 to 65 years-old, 18 were females and 2 were males. About 74 % (*n*=14/19) were employees or students in human or veterinary healthcare institutions, 31.6 % (*n*=6/19) underwent antimicrobial treatment in the last year and 5.3 % (*n*=1/19) were hospitalized in the previous year.

Among companion animals (*n*=29), 18 were males (12 dogs and 6 cats) and 11 were females (8 dogs and 3 cats) with ages ranging from 2 months to 17 years old.

All companion animals lived with their owners for at least 6 months prior to the sample collection, except one animal that had been adopted 1 month before (S16-cat). About 72 % (*n*=21/29, 20 dogs and 1 cat) of companion animals had access to outdoors. Hospitalization in the previous year was reported in 20.7 % (*n*=6/29, 2 dogs and 4 cats) of the companion animals. Only one dog (3.4%, *n*=1/29) had stayed in kennel/pet hotel in the last year. Contact with hospital environment was observed only in dogs (10.3 % of total sample, *n*=3/29). Around 17 % (*n*=5/29, 3 dogs and 2 cats) from companion animals from 4 households (S3, S4, S14 and S17) had been under antimicrobial treatment within the last year and amoxicillin-clavulanate was the antimicrobial prescribed. Moreover, the 2 cats were from the same household (S3) (Supplementary Table S2).

Antimicrobial resistance genes in fecal samples and bacteriological methods

Ten percent (*n*=2/20) and 70% (*n*=14/20) of the households concerning *Enterobacteriales* and staphylococci, respectively, lacked any of the tested antimicrobial resistance genes (AMRs) (Tables 1 and 2). Regarding humans from the control group (HC) in 15.8% (*n*=3/19) and in 47.4% (*n*= 9/19) no AMRs were detected in *Enterobacteriales* and staphylococci, respectively (Tables 3 and 4).

Regarding *Enterobacteriales* and staphylococci genes, companion animals were positive for bla_{TEM-1} (17.2%, *n*=5/29), bla_{DHA-1} (3.4%, *n*=1/29), *sul1* (3.4%, *n*=1), *sul2* (27.6 %, *n*=8), *sul3* (3.4%, *n*=1), *cmlA* (3.4%, *n*=1), *tet*(A) (10.3%, *n*=3), *aac(6´)Ib-cr* (10.3 %, *n*=3), *blaZ* (13.8 %,

n=4), *cat*$_{pC221}$ (6.9%, *n*=2), *tet*(M) (10.3%, n=3), *dfr*(G) and *dfr*(K) (6.9 %, n=2, both) genes (Table 5).

The presence of the *bla*$_{TEM-1}$ gene, was detected in 33.3% (*n*=9/27) of fecal samples from companion animal owners and in 42.1% (*n*=8/19) of humans from the control group. Interestingly, two households (S3 and S6) had positive *bla*$_{TEM-1B}$ companion animals and humans simultaneously (Table 1). All *bla*$_{TEM-1}$ genes sequences were identical to the *bla*$_{TEM-1B}$ gene framework in the coding and promoter (P3) regions.

The *bla*$_{SHV}$ family of genes was only detected in human participants (Table 5). Companion animal owners were positive for *bla*$_{SHV-1}$ (*n*=3) and *bla*$_{SHV-33}$ (n=2), while humans from the control group we positive for *bla*$_{SHV-1}$ (*n*=1), *bla*$_{SHV-186}$ (*n*=1) and *bla*$_{SHV-27}$ (*n*=1). Of note, *bla*$_{SHV-33}$ and *bla*$_{SHV-1}$ were shared between humans of the same household (S7 and S19, respectively) (Table 1). The AmpC β-lactamases family of genes, *bla*$_{CMY-2}$ and *bla*$_{DHA-1}$, were detected in humans from both groups (Table 5). There was no statistical difference between beta-lactamase carriage in companion animal owners and humans from the control group (p>0.05).

The *dfrAI* gene responsible for trimethoprim resistance was only detected in human samples while the aminoglycoside *aac(6')-Ib-cr* resistance gene was mostly detected in companion animals (Table 5). However, *aac(6')-Ib-cr* and *dfrAI* resistance genes detection had no statistical significant between groups (companion animals *vs* owners and all humans *vs* companion animals) (p>0.05). Companion animals and humans of both groups had similar (p>0.05) carriage of tetracycline *tet*(A*)* and chloramphenicol *cmlA* resistance genes (Table 5).

Detection of sulfonamide resistance genes (*sul1*, *sul*2 and *sul3*) were higher in humans from both groups. Detection of *sul1* gene was higher (p=0.048) in owners than in companion animals (Table 5). The *sul2* gene was the gene that more humans and companion animals shared within the same household (30%, *n*=6/20) (Table 1). Moreover, *blaZ, cat*$_{pCC221}$, *tet*(M), *dfr*(K) and *dfr*(G) genes was shared between companion animals and humans from the same household. The *blaZ* and *tet*(M) were the genes that more humans and companion animals shared within the same household (10.0%, *n*=2/20; both) (Table 2 and Table 5). Yet, no statistical significance for the resistance genes from *Staphylococcus* spp were detected between fecal samples from humans without daily contact with humans *vs* owners and companion animals and humans from the same household.

Humans and companion animals from ten out of the twenty households (50%) shared at least one antimicrobial resistance gene (Tables 1 and 2). Yet, fecal samples from owners showed higher antimicrobial resistance gene frequency and diversity than companion animals (Table 5).

About seventeen percent (*n*=5/29) of fecal samples from companion animals were positive for antimicrobial resistance genes associated with *Enterobacteriales* and staphylococci against at least to three different classes of antimicrobials (Tables 1 and 2).

The most common antimicrobial resistance genes combination in pets regarding *Enterobacteriales* was *bla*$_{TEM-1B}$-*sul2* (*n*=2, one dog, one cat) from different households. These pets were one dog that had been under antimicrobial treatment in the last year and also both dog and cat that were hospitalized in the previous year. Regarding companion animal owners, 14.8% (*n*=4/27) had the presence of antimicrobial resistance genes to at least three different antimicrobial classes regarding *Enterobacteriales* and *Staphylococcous* spp (Tables 1 and 2).

The remaining antimicrobial resistance genes studied, namely, carbapenemases and colistin plasmid-resistant, *mec*A and *tet*(K) genes were not detected in any fecal sample from humans or companion animals.

Furthermore, third-generation cephalosporin (3GC)-resistant, carbapenem-resistant and colistin -resistant *Enterobacteriales* and methicillin resistant staphylococci (MRS) were screened by bacteriological methods and no positive growth occur for Carbapenem-resistant and colistin -resistant *Enterobacteriales* and MRS (Tables 1- 4). Moreover, fecal samples that were positive for (3GC)-resistant *Enterobacteriales*, extended-spectrum β-lactamases (ESBLs)/ or cefalosporinases AmpC β-lactamases were detected by the direct Genomic DNA extraction and amplification from fecal samples (Tables 1-4).

Regarding the epidemiological survey, no statistical associations were found with the antimicrobial resistance genes present on the fecal samples from humans and companion animals.

Discussion

In the present study, we report the detection of 3GC-resistant, Carbapenem-resistant and colistin-resistant *Enterobacteriales* and methicillin resistant staphylococci (MRS) and the presence of antimicrobial resistance genes in the fecal samples of healthy companion animals and their human household members as well as of humans without daily contact with companion animals.

The relationship between humans and companion animals has changed over the years. Nowadays, companion animals live in a “relationship of mutualism” with their owners.[2] The anthropomorphizing of companion animals has led to changes in the behavior of owners towards them, with increasing conducts like kissing, licking, sharing food and sharing beds. Considering the shared environment of humans and companion animals, their close relationship, and the increased frequency of antimicrobial-resistant bacteria detected in humans and companion animals, new opportunities are created for interspecies transfer of antimicrobial resistance genes.[1, 2]

Antimicrobials are used extensively in human medicine, veterinary medicine, food-producing animals and agriculture.[22] In Portugal, β-lactams, such as penicillins, are the most prescribed antimicrobials in humans followed by macrolides, lincosamides, streptogramins,

quinolones, tetracyclines and sulfonamides-trimethoprim.[23] In Veterinary medicine, penicillins are also the most commonly prescribed antimicrobials in companion animals (dogs and cats), namely, amoxicillin and amoxicillin–clavulanate.[24] Yet, lincosamides, quinolones, macrolides, tetracyclines (doxycycline), nitroimidazoles and sulfonamides-trimethoprim are also used in small animals practice.[5,24] Several antimicrobials classes are used in humans and companion animals are the same, leading to an overlap of the detected antimicrobial resistance genes.[1,25] *Enterobacteriales* resistance to β-lactams is increasing in humans and in companion animals and there are no specific β-lactamases that are restricted only to animals or humans.[19] This seems to be in line with the results from this study. The β-lactamases that are disseminated in the *Enterobacteriales* family, especially the ESBLs and cephalosporinases of AmpC type are of particular clinical relevance.

In this study, the bla_{TEM} gene was the most frequent β-lactam-resistance gene in humans and companion animals, which is in agreement with previous studies.[26-28] In Portugal, the TEM-β-lactamase has also been detected in *Enterobacteriales* from food-producing animals and from commensal and clinical isolates.[7,27] The bla_{TEM} genes detected in this study (from companion animals and humans) had a similar promotor and coding region polymorphisms as the bla_{TEM-1B} (according to the Sutcliffe numbering system).[29] Furthermore, in two households, the bla_{TEM-1B} was present in co-living humans and companion animals. This finding may have resulted from a zoonotic transfer of bla_{TEM-1B} genes harbored in *Enterobacteriales*. Nevertheless, a common source of colonization could also be hypothesized since this resistance mechanism has been extensively detected in Portugal.[7, 27]

CTX-M β-lactamases are the current dominant type of ESBLs worldwide, having overpassed the TEM and SHV β-lactamases in Europe, both in humans and animals.[4,30] Furthermore, Portugal is among the European countries with the highest frequency of ESBL detection, mainly TEM and CTX-M.[31-32] However, in this study, only the ESBL SHV-27 gene was detected in a healthy human from the control group. The bla_{SHV-27} gene has been previously detect in clinical *K. pneumoniae, E. coli* and *Enterobacter cloacae* from humans and in clinical *K. pneumoniae* from dogs of different countries.[33-36] To the best of our knowledge, this is the first detection of bla_{SHV-27} gene in fecal samples from healthy humans in Portugal and in Europe. Moreover, in this human fecal sample we detected a 3GC-resistant *Klebsiella* spp.

Only the CMY-2 and DHA-1 beta-lactamases encoding genes were detected among all the AmpC cephalosporinases genes tested in this study, and these occurred mainly in humans. The bla_{CMY-2} gene and the bla_{DHA-1} gene were each detected in one healthy human without daily contact with companion animals. The bla_{CMY-2} gene was detected in two humans from the same household. The detection of these AmpC genes was already described in Portugal in clinical strains of *Enterobacteriales* from humans in the community and hospital, but as far as we are aware not in healthy individuals in the community.[37]

The frequency of sulphonamide resistance *sul* genes (*sul1*, *sul2* and *sul3*) here reported is in agreement with previous studies. [25,32-35] In Portugal, *sul1* and *sul2* resistance genes have been detected in commensal *Enterobacteriales* from fecal samples of healthy humans, food-producing animals and from clinical isolates.[32,40,41] Likely due to its dissemination, the *sul2* gene was most the frequent among humans and companion animals and was frequently detected in members of the same household.

The effect of the low antimicrobial consumption, the household controlled environment, and the possible food-borne dissemination of antimicrobial resistance genes should also be considered in this study as an explanation for the shared antimicrobial resistance genes.[36-39] The presence of ESBLs/AmpC in this study was lower than previously reported in healthy dogs in Portugal (Lisbon area).[4] In a previous study from our group, dogs from shelters/breeders were approximately three times more likely to have an ESBL/AmpC-producing *E. coli* than dogs from private owners.[4] The results in the present study may be explained by the fact that companion animals included in this study had little contact with kennels and where healthy.

This study showed that humans and companion animals carried and shared several antimicrobial resistance genes of clinical importance. Most of these genes are usually associated with mobile genetics elements (plasmids, integrons and transposons), which are important for antimicrobial resistance transfer between different microbiomes.[42-47]

The small sample size of this study is a limitation that may have limited the detection of ESBL/AmpC, carbapenemases, colistin plasmid-resistant genes and methicillin –resistant *Staphylococcus* spp. and the detection of statistical associations between the presence of antimicrobial resistance genes and specific risk factors. Moreover, in this study were detected different *dfr* genes (conferring resistance to trimethoprim), regarding *Staphylococus* spp. and usually the *dfr* genes found in coagulase-positive (CoPS) and coagulase-negative (CoNS) staphylococci are different. [49] In CoPS, the gene *dfr*(G) is the most common in *Staphylococcus pseudintermedius* isolates; while the *dfr*(K) gene is the most common in *Staphylococcus aureus*. [19, 48]

Nevertheless, the role of companion animals in the dissemination of clinically relevant antimicrobial resistance genes to humans through fecal contamination should not be neglected. Additionally, commensal *Enterobacteriales* and *Staphylococcu*s spp. of healthy humans may also be a reservoir for antibiotic-resistance determinants.[1, 49]

Further studies are needed to determine the causality and directionality of resistance genes transfer between human and companion animals, in order to identify the critical control points at which interventions could substantially prevent the spread of antimicrobial resistance genes within households and establish the prevention and intervening measures for controlling resistance.

In this study, were implemented a rapid easy methodology, which easily detected antimicrobial resistant genes that are of particular interest to epidemiological studies. Highly discriminatory universal methods, such whole- genome -sequencing, are expensive to the most of the laboratories. We also validated its usefulness in situations requiring rapid MRS, ESBLs/AmpC, carbapenemases- producing and colistin plasmid- resistant *Enterobacteriales*.

The combination of molecular techniques with culture methods should be pursued in the future to increase the detection of antimicrobial resistance determinants leading to a better understanding of the overlap between the human and companion animal gut resistome.

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Table 1: ESBLs/ AmpC, Carbapenemases - producing and colistin plasmid-resistant *Enterobacteriales* screening and antimicrobial resistant genes in fecal samples from companion animals and humans from households

Household	Specie	MCK + CTX Bacteria (CFU/g)	MCK + MEM Bacteria (CFU/g)	SuperPolymyxin medium	β-lactams - resistant genes	Sulfonamides - resistant genes	Trimethoprim-resistant genes	Aminoglycoside-resistant genes	Tetracyclines-resistant genes	Chloramphenicol - resistant genes	Colistin plasmid-resistant genes
S1	Human1	no growth	no growth	no growth	-	-	-	-	-	-	-
	Human2	no growth	no growth	no growth	*bla*$_{TEM-1B}$	*sul3*	-	-	-	*cmlA*	-
	Human3	no growth	no growth	no growth	*bla*$_{TEM-1B}$	*sul2*	-	-	-	*cmlA*	-
	Dog1	no growth	no growth	no growth	-	-	-	*aac(6')-Ib-cr*	-	-	-
	Dog2	no growth	no growth	no growth	-	-	-	-	-	-	-
S2	Human1	no growth	no growth	[a] intrinsic bacterial species	-	-	*dfrIa*	-	-	-	-
	Cat1	no growth	no growth	no growth	-	-	-	-	-	-	-
S3	Human1	no growth	no growth	no growth	*bla*$_{TEM-1B}$	-	**-**	-	*tet*(A)	**-**	-
	Cat1	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$	*sul2*	*dfrIa*	**-**	**-**	**-**	-
	Cat2	no growth	no growth	[a] intrinsic bacterial species	**-**	*sul2*	**-**	**-**	**-**	**-**	-
S4	Human1	no growth	no growth	[a] intrinsic bacterial species	**-**	*sul2*	**-**	**-**	**-**	**-**	-
	Dog1	no growth	no growth	no growth	*bla*$_{TEM-1B}$	*sul2*	**-**	-	*tet*(A)	**-**	-
S5	Human1	no growth	no growth	no growth	*bla*$_{SHV-1}$	*sul2*	**-**	**-**	**-**	**-**	-
	Cat1	no growth	no growth	no growth	**-**	-	**-**	**-**	**-**	**-**	**-**
S6	Human1	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$	*sul2, sul3*	-	-	*tet*(A)	*cmlA*	-

	Cat1	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$	*sul2*	-	-	*tet*(A)	-	-
S7	Human1	*E. coli* (5X10^3)	no growth	[a] intrinsic bacterial species	*bla*$_{SHV-33}$, *bla*$_{CMY-2}$	*sul1*	-	-	*tet*(A)	-	-
	Human2	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$, *bla*$_{SHV-33}$	*sul1*	-	-	*tet*(A)	-	-
	Human3	*E. coli* (6X10^3)	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$, *bla*$_{CMY-2}$	*sul1*, *sul2*	-	-	*tet*(A)	-	-
	Cat1	no growth	no growth	no growth	**-**	*sul2*	**-**	**-**	**-**	**-**	**-**
S8	Human1	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$	*sul1*, *sul2*	*dfrIa*	*aac(6')-Ib-cr*	**-**	**-**	
	Dog1	no growth	no growth	no growth	**-**	*sul2*	**-**	**-**	**-**	**-**	**-**
S9	Human1	no growth	no growth	[a] intrinsic bacterial species	**-**	*sul2*	**-**	**-**	**-**	**-**	**-**
	Cat1	no growth	no growth	no growth	**-**	*sul2*	**-**	**-**	-	*cmlA*	**-**
S10	Human1	no growth	no growth	[a] intrinsic bacterial species	**-**	-	**-**	**-**	**-**	**-**	**-**
	Dog1	*Klebsiella* spp.*	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$, *bla*$_{DHA-1}$	*sul3*	**-**	-	*tet*(A)	**-**	**-**
S11	Human1	no growth	no growth	[a] intrinsic bacterial species	**-**	-	**-**	**-**	**-**	**-**	**-**
	Dog1	no growth	no growth	[a] intrinsic bacterial species	**-**	-	**-**	**-**	**-**	**-**	**-**
S12	Human1	no growth	no growth	[a] intrinsic bacterial species	**-**	*sul2*	**-**	-	*tet*(A)	**-**	**-**
	Dog1	no growth	no growth	[a] intrinsic bacterial species	**-**	*sul2*	**-**	**-**	**-**	**-**	**-**

S13	Human1	no growth	no growth	[a] intrinsic bacterial species	-	*sul*1	-	-	-	-	-
	Dog1	no growth	no growth	[a] intrinsic bacterial species	-	*sul*1	-	-	-	-	-
	Dog2	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
S14	Human1	no growth	no growth	no growth	-	-	-	-	-	-	-
	Human2	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
	Dog1	no growth	no growth	no growth	-	-	-	-	-	-	-
	Dog2	no growth	no growth	no growth	bla_{TEM-1B}	-	-	-	-	-	-
	Dog3	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
S15	Human1	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
	Dog1	no growth	no growth	no growth	-	-	-	-	-	-	-
	Dog2	no growth	no growth	no growth	-	-	-	-	-	-	-
	Cat1	no growth	no growth	no growth	-	-	-	-	-	-	-
S16	Human1	no growth	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}	-	-	-	-	-	-
	Human2	no growth	no growth	[a] intrinsic bacterial species	-	*sul*2	-	-	*tet*(A)	-	-
	Dog1	no growth	no growth	[a] intrinsic bacterial species	-	-	-	*aac(6')-Ib-cr*	-	-	
	Cat1	no growth	no growth	no growth	-	-	-	-	-	-	
S17	Human1	no growth	no growth	[a] intrinsic bacterial species	-	*sul2*	-	-	-	-	-

	Dog1	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
S18	Human1	no growth	no growth	[a] intrinsic bacterial species	-	*sul1*	-	-	-	-	-
	Dog1	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
S19	Human1	no growth	no growth	[a] intrinsic bacterial species	$bla_{TEM-1B,}$ bla_{SHV-1}	-	*dfrIa*	-	-	-	-
	Human2	no growth	no growth	[a] intrinsic bacterial species	bla_{SHV-1}	-	-	-	-	-	-
	Dog1	no growth	no growth	no growth	-	-	-	-	-	-	-
S20	Human1	no growth	no growth	no growth	bla_{SHV-1}	-	-	-	-	-	-
	Dog1	no growth	no growth	no growth	-	-	-	-	-	-	-
	Dog1	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-

Legend: CFU- colony-forming unit; MCK+CTX - MacConkey agar plates with 1.5 µg/ml of cefotaxime; MCK+MEM: MacConkey agar plates with 1.5 µg/ml of meropenem; [a] : *Proteus* spp. and/or *Serratia* spp . (polymyxin resistance intrinsic bacterial species) [21] ; *obtained from the enrichment medium; -: not detected.

Table 2: Methicillin-resistant *Staphylococcus* spp screening and antimicrobial resistance genes detected from *Staphylococcus spp* in fecal samples from companion animals and humans from households

Household	Specie	Brilliance™ MRSA plates	β-lactams - resistant genes	Chloramphenicol - resistant genes	Tetracyclines - resistant genes	Trimethoprim - resistant genes
S1	Human1	no growth	-	-	*tet*(M)	-
	Human2	no growth	*blaZ*	cat_{pC221}	-	*dfr*(K)
	Human3	no growth	-	-	*tet*(M)	-
	Dog1	no growth	-	-	-	-
	Dog2	no growth	*blaZ*	cat_{pC221}	*tet*(M)	*dfr*(K)
S2	Human1	no growth	-	-	-	-
	Cat1	no growth	-	-	-	-
S3	Human1	no growth	-	-	-	-
	Cat1	no growth	-	-	-	-
	Cat2	no growth	-	-	-	-
S4	Human1	no growth	-	-	-	-
	Dog1	no growth	-	-	-	-
S5	Human1	no growth	-	-	-	-
	Cat1	no growth	*blaZ*	cat_{pC221}	-	*dfr*(K)
S6	Human1	no growth	-	-	-	-
	Cat1	no growth	-	-	-	-
	Human1	no growth	-	-	-	-

S7	Human2	no growth	-	-	-	-
	Human3	no growth	-	-	-	-
	Cat1	no growth	**-**	**-**	**-**	**-**
S8	Human1	no growth	-	**-**	-	-
	Dog1	no growth	**-**	**-**	**-**	**-**
S9	Human1	no growth	**-**	**-**	**-**	**-**
	Cat1	no growth	**-**	**-**	**-**	**-**
S10	Human1	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	-	**-**
S11	Human1	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	**-**	**-**
S12	Human1	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	**-**	**-**
S13	Human1	no growth	*blaZ*	**-**	*tet*(M)	*dfr*(G)
	Dog1	no growth	*blaZ*	**-**	*tet*(M)	*dfr*(G)
	Dog2	no growth	*blaZ*	**-**	*tet*(M)	*dfr*(G)
S14	Human1	no growth	**-**	**-**	**-**	**-**
	Human2	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	**-**	**-**
	Dog2	no growth	**-**	**-**	**-**	**-**
	Dog3	no growth	**-**	**-**	**-**	**-**

S15	Human1	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	**-**	**-**
	Dog2	no growth	**-**	**-**	**-**	**-**
	Cat1	no growth	**-**	**-**	**-**	**-**
S16	Human1	no growth	**-**	**-**	**-**	**-**
	Human2	no growth	*blaZ*	cat_{pC221}	*tet*(M)	*dfr*(G)
	Dog1	no growth	**-**	**-**	**-**	**-**
	Cat1	no growth	**-**	**-**	**-**	**-**
S17	Human1	no growth	**-**	**-**	*tet*(M)	**-**
	Dog1	no growth	-	**-**	**-**	**-**
S18	Human1	no growth	**-**	**-**	**-**	**-**
	Dog1	no growth	**-**	**-**	**-**	**-**
S19	Human1	no growth	**-**	-	*tet*(M)	**-**
	Human2	no growth	*blaZ*	**-**	*tet*(M)	*dfr*(G), *dfr*(K)
	Dog1	no growth	-	**-**	**-**	**-**
S20	Human1	no growth	-	-	-	-
	Dog1	no growth	-	-	-	-
	Dog1	no growth	-	-	-	-

Legend: -: not detected

Table 3: ESBLs/ AmpC, Carbapenemases - producing and colistin plasmid-resistant *Enterobacteriales* screening and antimicrobial resistant genes from fecal human samples without daily contact with companion animals.

Human	MCK + CTX Bacteria (CFU/g)	MCK + MEM Bacteria (CFU/g)	SuperPolymyxin medium	β-lactams-resistant genes	Sulfonamides-resistant genes	Trimethoprim-resistant genes	Aminoglycoside - resistant genes	Tetracyclines-resistant genes	Chloramphenicol - resistant genes	Colistin plasmid-resistant genes
H1	no growth	no growth	[a] intrinsic bacterial species	-	*sul2*	*dfrIa*	-	-	-	-
H2	*E. coli**	no growth	[a] intrinsic bacterial species	bla_{CMY-2}	*sul2, sul3*	-	-	-	-	-
H3	no growth	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}	*sul2*	-	-	-	*cmlA*	-
H4	no growth	no growth	no growth	bla_{TEM-1B}	-	-	-	-	-	-
H5	no growth	no growth	no growth	-	*sul1*	-	-	-	-	-
H6	no growth	no growth	no growth	bla_{TEM-1B}	*sul2*	-	-	-	-	-
H7	*Klebsiella* spp ($3X10^3$)	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}, bla_{SHV-1}, bla_{DHA-1}	*sul1, sul3*	*dfrIa*	-	*tet*(A)	-	-
H8	no growth	no growth	[a] intrinsic bacterial species	-	*sul1, sul2, sul3*	-	-	*tet*(A)	*cmlA*	-
H9	no growth	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}	*sul2*	-	-	*tet*(A)	-	-
H10	no growth	no growth	[a] intrinsic bacterial species	-	*sul1*	-	-	-	-	-
H11	no growth	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}, $bla_{SHV-186}$	-	-	-	-	-	-
H12	*Klebsiella* spp. ($1X10^3$)	no growth	[a] intrinsic bacterial species	bla_{TEM-1B}, bla_{SHV-27}	*sul3*	-	-	-	-	-

H13	no growth	no growth	[a] intrinsic bacterial species	*bla*$_{TEM-1B}$	-	-	-	-	-	-
H14	no growth	no growth	[a] intrinsic bacterial species	-	-	-	*aac(6')-Ib-cr*	-	-	-
H15	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	*cmlA*	-
H16	no growth	no growth	[a] intrinsic bacterial species	-	*sul1*	-	-	-	-	-
H17	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
H18	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-
H19	no growth	no growth	[a] intrinsic bacterial species	-	-	-	-	-	-	-

Legend: CFU- colony-forming unit; MCK+CTX - MacConkey agar plates with 1.5 µg/ml of cefotaxime; MCK+MEM: MacConkey agar plates with 1.5 µg/ml of meropenem; * obtained from the enrichment medium; [a] - *Proteus* spp. and/or Serratia spp. (polymyxin resistance intrinsic bacterial species) [21]; -: not detected.

Table 4: Methicillin-resistant *Staphylococcus* spp. screening and antimicrobial resistance genes detected from *Staphylococcus* spp. in fecal human samples without daily contact with companion animals.

Human	Brilliance ™ MRSA plates	*β-lactams* - resistant genes	Chloramphenicol resistant genes	Tetracyclines resistant genes	Trimethoprim resistant genes
H1	no growth	*blaZ*	cat_{pC221}	*tet*(M)	*dfr*(K)
H2	no growth	*blaZ*	cat_{pC221}	*tet*(M)	*dfr*(K)
H3	no growth	-	-	*tet*(M)	-
H4	no growth	-	-	-	-
H5	no growth	-	-	-	-
H6	no growth	-	-	-	-
H7	no growth	-	-	*tet*(M)	-
H8	no growth	-	-	*tet*(M)	-
H9	no growth	-	-	-	-
H10	no growth	-	-	-	-
H11	no growth	-	-	*tet*(M)	-
H12	no growth	-	-	-	-
H13	no growth	-	-	-	*dfr*(K)
H14	no growth	-	-	*tet*(M)	-
H15	no growth	-	-	*tet*(M)	-
H16	no growth	-	-	*tet*(M)	-
H17	no growth	-	-	-	-
H18	no growth	-	-	-	-
H19	no growth	-	-	-	-

Legend: -: not detected

Table 5: Antimicrobial resistance genes frequency in fecal samples from companion animals and humans.

Genes	Human (control, n=19)	Human (Owners, n=27)	Companion animals (n=29)	Household[a] Human-Animal sharing	Household Human-Human sharing	Household Animal-Animal sharing
	n, (%)	n, (%)	n, (%)	n, (%)	n, (%)	n, (%)
bla$_{SHV}$group	3, (15.8)	6, (22.2)	0, (0.0)	0, (0.0)	2, (10.0)	0, (0.0)
bla$_{TEM}$group	7, (36.8)	9, (33.3)	5, (17.2)	2, (10.0)	2, (10.0)	0, (0.0)
bla$_{DHA-1}$	1, (5.3)	0, (0.0)	1, (3.4)	0, (0.0)	0, (0.0)	0, (0.0)
bla$_{CMY-2}$	1, (5.3)	2, (7.4)	0, (0.0)	0, (0.0)	1, (5.0)	0, (0.0)
sul1	6, (31.6)	6, (22.2)	1, (3.4)	1, (5.0)	1, (5.0)	0, (0.0)
*sul*2	7, (36.8)	12, (44.4)	8, (27.6)	6, (30.0)	1, (5.0)	1, (5.0)
*sul*3	4, (21.1)	2, (7.4)	1, (3.4)	0, (0.0)	0, (0.0)	0, (0.0)
*dfr*Ia	2, (10.5)	4, (14.8)	0, (0.0)	0, (0.0)	0, (0.0)	0, (0.0)
cmlA	4, (21.1)	3, (11.1)	1, (3.4)	0, (0.0)	1, (5.0)	0, (0.0)
*tet*A	4, (21.1)	7, (25.9)	3, (10.3)	1, (5.0)	1, (5.0)	0, (0.0)
aac(6´)Ib-cr	2, (10.5)	0, (0.0)	3, (10.3)	0, (0.0)	0, (0.0)	0, (0.0)
blaZ	2, (10.5)	4, (14.8)	4, (13.8)	2, (10.0)	1, (5.0)	1, (5.0)
cat pC221	2, (10.5)	2, (7.4)	2, (6.9)	1, (5.0)	0, (0.0)	0, (0.0)
tet(M)	9, (47.4)	7, (25.9)	3, (10.3)	2, (10.0)	2, (10.0)	1, (5.0)
dfr(G)	0, (0.0)	3, (11.1)	2, (6.9)	1, (5.0)	0, (0.0)	1, (5.0)
dfr(K)	3, (15.8)	2, (7.4)	2, (6.9)	1, (5.0)	0, (0.0)	0, (0.0)

[a] Total number of households is 20.

Supplementary Table 1: Oligonucleotides used in this study.

Target gene	Primer name	Sequence (5'–3')	PCR product size (bp)	Reference
16S rRNA	16S-F	GTGCCAGCAGCCGCGGTAA	886	6
	16S-R	AGACCCGGGAACGTATTCAC		
*bla*TEM	FIN	ATTCTTGAAGACGAAAGGGC	1092	11
	DEB	ATGAGTAAACTTGGTCTGAC		
*bla*SHV	SHV-F	CGCTTCTTTACTCGCCTTTA	910	11
	SHV-R	TTACGCTTGCCAGTGCTC		
*bla*CTX-M-1	CTX-M-Group 1-F	AAAAATCACTGCGCCAGTTC	415	10
group	CTX-M-Group 1-R	AGCTTATTCATCGCCACGTT		
*bla*CTX-M-2	CTX-M-Group 2-F	CGACGCTACCCCTGCTATT	552	10
group	CTX-M-Group 2-R	CCAGCGTCAGATTTTTCAGG		
*bla*CTX-M-9	CTX-M-Group 9-F	CAAAGAGAGTGCAACGGATG	205	10
group	CTX-M-Group 9-R	ATTGGAAAGCGTTCATCACC		
blaMOX-1, MOX-2, CMY-1, CMY-8 to CMY-11	MOXMF	GCTGCTCAAGGAGCACAGGAT	520	12
	MOXMR	CACATTGACATAGGTGTGGTGC		
blaLAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITMF	TGGCCAGAACTGACAGGCAAA	462	12
	CITMR	TTTCTCCTGAACGTGGCTGGC		
blaDHA-1, DHA-2	DHAMF	AACTTTCACAGGTGTGCTGGGT	405	12
	DHAMR	CCGTACGCATACTGGCTTTGC		
*bla*ACC	ACCMF	AACAGCCTCAGCAGCCGGTTA	346	12
	ACCMR	TTCGCCGCAATCATCCCTAGC		
*bla*MIR-1T,	EBCMF	TCGGTAAAGCCGATGTTGCGG	302	12
ACT-1	EBCMR	CTTCCACTGCGGCTGCCAGTT		
blaFOX-1 to FOX-5b	FOXMF	AACATGGGGTATCAGGGAGATG	190	12
	FOXMR	CAAAGCGCGTAACCGGATTGG		
blaCMY-2	CMY- F	ATGATGAAAAAATCGTTATGC	1146	4
	CMY- R	TTATTGYAGCTTTTCAAGAATGC		
blaDHA-1	DHA-1-F	TTATTCCAGTGCACTCAAAA	1140	4
	DHA-1-R	ATGAAAAAATCGTTATCTGCAACACTG		
*bla*IMP	IMP-F	GGAATAGAGTGGCTTAAYTCTC	232	13
	IMP-R	GGTTTAAYAAAACAACCACC		
*bla*SPM	SPM-F	AAAATCTGGGTACGCAAACG	271	13
	SPM-R	ACATTATCCGCTGGAACAGG		
*bla*VIM	VIM-F	GATGGTGTTTGGTCGCATA	390	13
	VIM-R	CGAATGCGCAGCACCAG		
*bla*OXA	OXA-F	GCGTGGTTAAGGATGAACAC	438	13
	OXA-R	CATCAAGTTCAACCCAACCG		
*bla*BIC	BIC-F	TATGCAGCTCCTTTAAGGGC	537	13

	BIC-R	TCATTGGCGGTGCCGTACAC		
*bla*NDM	NDM-F	GGTTTGGCGATCTGGTTTTC	621	13
	NDM-R	CGGAATGGCTCATCACGATC		
*bla*KPC	KPC-F	CGTCTAGTTCTGCTGTCTTG	798	13
	KPC-R	CTTGTCATCCTTGTTAGGCG		
sul1	Sul1-F	TGGTGACGGTGTTCGGCATTC	789	14
	Sul1-R	GCGAGGGTTTCCGAGAAGGTG		
sul2	Sul2-F	CGGCATCGTCAACATAAC	722	14
	Sul2-R	GTGTGCGGATGAAGTCAG		
sul3	sul3-F	GAGCAAGATTTTTGGAATCG	900	16
	Sul3-R	CATCTGCAGCTAACCTAGGGCTTTGGA		
dfrIa	DfrIa-F	GTGAAACTATCACTAATGG	474	14
	DfrIa-R	TTAACCCTTTTGCCAGATTT		
aac(6')-Ib	AAC6Ib-F	TTGCGATGCTCTATGAGTGG	482	15
	AAC6Ib-R	CTCGAATGCCTGGCGTGTTT		
tet(A)	TetA - F	GTAATTCTGAGCACTGTCGC	937	14
	TetA - R	CTGCCTGGACAACATTGCTT		
cmlA	cmlA - F	TGTCATTTACGGCATACTCG	456	14
	cmlA - R	ATCAGCCATCCCATTCCCAT		
mcr-1	*mcr1*_320bp - F	AGTCCGTTTGTTCTTGTGGC	320	17,18
	*mcr1*_320bp - R	AGATCCTTGGTCTCGGCTTG		
mcr-2	*mcr2*_700bp - F	CAAGTGTGTTGGTCGCAGTT	715	17,18
	*mcr2*_700bp - R	TCTAGCCCGACAAGCATACC		
mcr-3	*mcr3*_900bp - F	AAATAAAAATTGTTCCGCTTATG	929	17,18
	*mcr3*_900bp - R	AATGGAGATCCCCGTTTTT		
mcr-4	*mcr4*_1100bp - F	TCACTTTCATCACTGCGTTG	1116	17,18
	*mcr4*_1100bp - R	TTGGTCCATGACTACCAATG		
mcr-5	*MCR5* - F	ATGCGGTTGTCTGCATTTATC	1644	17,18
	MCR5 - R	TCATTGTGGTTGTCCTTTTCTG		
*mec*A	mecA - F	GGGATCATAGCGTCATTATTC	527	6
	mecA - R	AACGATTGTGACACGATAGCC		
blaZ	blaZ - F	CAGTTCACATGCCAAAGAG	772	19
	blaZ - R	TACACTCTTGGCGGTTTC		
cat pC221	catpC221 - F	ATTTATGCAATTATGGAAGTTG	435	19
	catpC221 - R	TGAAGCATGGTAACCATCAC		
tet(M)	tetM - F	GTTAAATAGTGTTCTTGGAG	656	19
	tetM - R	CTAAGATATGGCTCTAACAA		
tet(K)	tetK - F	TTAGGTGAAGGGTTAGGTCC	718	19
	tetK - R	GCAAACTCATTCCAGAAGCA		
dfr(G)	dfrG - F	TTTCTTTGATTGCTGCGATG 3	501	19
	dfrG - R	AACGCACCCGTTAACTCAAT		
dfr(K)	dfrK - F	GCTGCGATGGATAAGAACAG	314	20
	dfrK - R	GGACGATTTCACAACCATTAAAGC		

F, sense primer; R, antisense primer.

Supplementary Table 2: Enrollment and questionnaire analyses

Epidemiological questionnaire	Human (control, n=19) n, (%)	Human (Owners, n=27) n, (%)	Companion animals (n=29) n, (%)
Age (Years)	22-65	8-66	0.17-17
Gender			
Males	1, (5.3)	7 (25.9)	18, (62.1)[a]
Females	18, (94.7)	20 (74.1)	11 (37.9)[b]
Contact with hospital environment	na	na	3 (10.3)[c]
Antimicrobial use in last year	6, (31.6)	9, (33)	5, (17%.0)[d]
Hospitalization in the last year	1, (5.3)	3, (11.1)	6, (20.7)[e]
kennel/hotel access	na	na	1 (3.4)
Lifestyle -Outdoor	na	na	21, (72.0)[f]
Employee or student in human or veterinary healthcare institutions	14, (19.0)	14, (52.0)	na

Legend: [a] 12 dogs and 6 cats; [b] 8 dogs and 3 cats; [c] only dogs **;** [d] 3 dogs and 2 cats; [e] 20 dogs and 1 cat**;** [f] 2 dogs and 4 cats; na- not applicable.

4.1.2- OXA-181-Producing Extraintestinal Pathogenic *Escherichia coli* Sequence Type 410 Isolated from a Dog in Portugal

Short-form paper published at *Antimicrobial Agents and Chemotherapy Journal*.

Brilhante, M., Menezes, J., **Belas A.,** Pomba C., Vincent Perreten. 2020. OXA-181-producing extra-intestinal pathogenic *Escherichia coli* ST410 isolated from a dog in Portugal. Antimicrobial Agents and Chemotherapy Journal. pii: AAC.02298-19. doi: 10.1128/AAC.02298-19.

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One Poster communication at the International congress 29th ECCMID, 2019, Amsterdam, Netherlands.

OXA-181-producing Extra-intestinal Pathogenic *Escherichia coli* ST410 isolated from a dog in Portugal.

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Running title: *bla*OXA-181-producing ExPEC belonging to ST410 isolated from a pet

Abstract

Two multidrug-resistant and carbapenemase-producing *Escherichia coli* clones of ST410 were isolated from fecal samples of a dog with skin infection at admission to an animal hospital in Portugal, and one month after discharge. Whole-genome sequencing revealed a 126,409-bp Col156/IncFIA/IncFII multidrug-resistance plasmid and a 51,479-bp IncX3 $bla_{OXA-181}$-containing plasmid. The chromosome and plasmids carried virulence genes characteristic for uropathogenic *E. coli* indicating that dogs may carry multidrug-resistant *E. coli* related to those causing UTI in humans.

Keywords: Pets, carbapenemase, veterinary, ExPEC, companion animals.

Carbapenemase-producing *Enterobacteriaceae* (CPE) represent a major public health issue and the frequency of their detection in pets has been increasing worlwide (1). A predominant carbapenemase- producing Escherichi coli associated so far with infections in humans belonged to specific clonal lineages (2). Among them, the high-risk multidrug-resistant (MDR) sequence type (ST) 410 shows strong potential for transmission between different hosts including companion animals (CAs) and humans (1, 3). CPEc ST410 has been identified in 2016 in United Kingdom (4), in 2017 in Korea (5) and 2018 in Switzerland (6), and mostly associated with nosocomial carriage (6). Because of this CPE emergence in CAs and their potential transmission to humans (3, 7), CPE gut carriage was assessed in: (i) healthy CAs from the community, (ii) CAs with urinary tract infections (UTIs), and (iii) CAs suffering from skin/soft tissue infections (SSTIs) attending the University Veterinary Teaching Hospital (UVTH) in Lisbon, Portugal.

Fecal samples of 71 healthy CAs (47 dogs and 24 cats) were collected at home, whilst those of CAs with UTIs (13 dogs and 2 cats) and 12 CAs with SSTIs (11 dogs and 1 cat) were taken at admission to UVTH for CPE screening between January 2016 and August 2019. Signed informed consent from the owners and ethical approval were obtained (CEBEA 027/2018). Samples were plated, with and without pre-enrichment in peptone water, onto MacConkey agar plates supplemented with antibiotic discs containing meropenem (10µg), temocillin (30µg) and CAT-ID™ (mastdiscs™ ID for CPE screening). Isolates were identified using MALDI-TOF (Brucker). Carbapenemase production was assessed using Blue-carba (8). One dog from the SSTI group was positive in 2017 for CPEc (strain PT113) at admission and was still positive after 1 month (strain PT109). PT109 and PT113 were non-susceptible to ampicillin (64 µg/mL), ceftazidime (>128 µg/mL), cefotaxime (>64 µg/mL), cefepime (>32 µg/mL), ciprofloxacin (>8 µg/mL), chloramphenicol (16 µg/mL), ertapenem (1 µg/mL), sulfamethoxazole (>1024 µg/mL), trimethoprim (>32 µg/mL), and tetracycline (>64 µg/mL) as determined by broth microdilution (EUVSEC/EUVSEC2, Thermo Fisher Scientific) according to CLSI recommendations (9).

The genomic sequence of PT109 was obtained using both MinION R.9.4.1 flow cell (Oxford Nanopore Technology, ONT) and Novaseq6000 (Illumina), and assembled with Unicycler (v.0.4.4), whilst PT113 was sequenced with Novaseq6000, readmapped to contigs of PT109 (Geneious v10.1.3) and assembled using SPAdes (v3.12.0). The full assembly of PT109 resulted in the 4,815,030-bp chromosome (GenBank accession number CP041031), the 126,409-bp Col156/IncFIA/IncFII plasmid pLB_CTX-M-15 (CP041032), and the 51,479-bp IncX3 plasmid pLB_OXA-181 (CP041033), and the the 126,409-bp Col156/IncFIA/IncFII plasmid pLB_CTX-M-15 (CP041032), and the 51,479-bp IncX3 plasmid pLB_OXA-181 (CP041033).

PlasmidFinder 2.1, Resfinder 3.2, MLST, SerotyFinder 2.0, Virulence Finder 2.0, (http://wwwgenomicepidemiology.org/), virulence factor database

(http://wwwmgc.ac.cn/VFs/main.htm), ISFinder (https://isfinder.biotoul.fr/) and INTEGRALL (http://integral.bio.ua.pt/) were used for *in silico* analyses.

PT109 and PT113 belonged to ST410 and their serotype to the recently described genotype OgN5 (10). The colanic acid operon was upstream of the *O*-locus, which is crucial for biofilm production, withstanding desiccation (11) and for protection against complement-mediated killing in serum (12). They contained amino acid substitution associated with fluoroquinolone resistance in GyrA (S83L and D87N) and ParC (S80I) as well as multiple antimicrobial resistance genes (ARGs) on plasmids pLB_CTX-M-15 and pLB_OXA-181 (Figure 1). Plasmid pLB_OXA-181 was virtually identical to other OXA-181-containing IncX3 plasmids in the NCBI database (one SNP difference) (5) (Figure 1). The two plasmids sharing the closest similarity to pLB_CTX-M-15 lack either the resistance integron In*54* or the iron transport systems (Figure 1).

The genome of PT109 and PT113 exhibited a repertoire of virulence factors (n=25) (Table 1) that classifies them as extraintestinal pathogenic *E. coli* (ExPEC) (13). Specifically, twelve of them are characteristic for uropathogenic *E. coli* (UPEC) (Table 1). UPEC virulence factors like the iron-related systems SitABCD, aerobactin (*iucABCD*, *iutA*) and CjrABC-SenB, and the arginine deiminase operon (ADO, *arcACBDR*) were all located on pLB_CTX-M-15 (Figure 1). The *cjrABC-senB* gene cluster is involved in the virulence of UPEC in humans (14) and ADO enhanced the capacity of a wild-type strain of *E. coli* to infect kidneys in a mouse model (15), indicating that PT109 and PT113 have strong potential for developing UTIs in humans (13-15).

Comparative analysis of virulence and ARGs found in PT109 and PT113 with those of other ST410 strains from the NCBI showed that they all contained different ARGs but shared equally twelve of the virulence factors (Supplementary Tables S1 and S2). However, presence of the Sit operon (40% of strains) and aerobactin was less common (35%), whilst the colanic acid operon (25%), yersiniabactin (21%), and CjrABC-SenB (4%) were rarely detected (Supplementary Table S2). In fact, none of these other strains possessed the same virulence and resistance traits as PT109 and PT113, emphasizing the genetic plasticity and the variable pathogenic potential of the ST410 lineage. These different factors might have contributed to the colonization success and persistence capacity of PT109 and PT113, which cannot be explained by antibiotic selective pressure alone.

The genetic relationship among PT109, PT113 and other ST410 strains from the GenBank database (n=26) was also determined using an *ad-hoc* core genome MLST analysis comparing 3375 genes, common to all strains (cgMLST Target Definer, Seqsphere+ v6.0.2). The resulting phylogenetic analysis dissociated PT109 and PT113 from the other CPEc of animal origin, but connected them to human strains isolated from urine, which supports the assumption that both strains have potential for developing UTIs in humans (Figure 2). PT109 and PT113 were highly genetically related, differing by only one allele, whereas they differed from the other

ST410 strains by at least 23 and up to 262 different alleles. Strains from the same clade of PT109 and PT113 (CP018965, CP035325, CP032426 and CP035123) shared most virulence factors (n=24/26) and contained the same genotype OgN5, suggesting that specific ST410 lineages are more likely to be successful in colonization and/or infection.

This study provided an in-depth characterization of the first OXA-181-producing ExPEC obtained in a veterinary environment and its comparison with other ST410 strains. Detection of the same clone within a 1-month period indicates that such a MDR and carbapenemase-producing pathogenic *E. coli* can temporarily persist in dogs and disseminate into the environment, other animals and humans, therefore posing a major One Health concern (16, 17).

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Table 1. Virulence factors of *Escherichia coli* PT109 and PT113.

Virulence factor	Function (ref)	Pathotype [b]	Virulence gene(s)	Genetic location in PT109, coordinates (Accession number)
F9 fimbriae	Adherence (18)	AIEC, EAEC, EPEC, EHEC UPEC	*z2200-z2206*	Chromosome 2'432'071-2'438'432 (CP041031)
Hemorrhagic E. coli pilus	Adherence (19)	EHEC, ETEC, EPEC	*hcpABC*	Chromosome 3'881'323-3'884'350 (CP041031)
CFA/I fimbriae	Adherence (20)	ETEC	*cfaABCD*	Chromosome 691'527-696'541(CP041031)
Curli fibers	Adherence (13)	UPEC, SEPEC, APEC	*csgBAC, csgDEFG*	Chromosome 2'883'283-2'887'724 (CP041031)
Intimin-like FdeC	Adherence (21	ExPEC, UPEC, ETEC, EHEC, EPEC, AIEC, EAEC, STEC	*eaeH*	Chromosome 3'690'607-3'694'863 (CP041031)
E. coli common pilus	Adherence (22)	EPEC and commensals	*ecpRABCDE*	Chromosome 3'697'931-3'704'724 (CP041031)
Type 1 fimbriae	Adherence (13)	ExPEC, UPEC, NMEC, SEPEC, APEC	*fimBEAICDFGH*	Chromosome 4'135'565-4'144'317 (CP041031)
Stg fimbriae	Adherence (23)	ExPEC, APEC	*stgABCD*	Chromosome 4'783'515-4'788'497 (CP041031)
UpaG adhesin	Adherence (24)	ExPEC, UPEC	*upaG*	Chromosome 135'019-130'169 (CP041031)
E. coli laminin-binding fimbriae	Adherence (25)	EPEC, STEC, EHEC and commensals	*elfADCG*	Chromosome 2'982'660-2'989'604 (CP041031)
NlpI lipoprotein	Adherence(26)	EPEC, AIEC	*nlpI*	Chromosome 627'338-628'222 (CP041031)

Flagella	Adherence and Motility (27)	EPEC, ExPEC	*fliRQPONMLKJLHGFE*, *fliSTDC*, *fliAZY*, *flhDC*, *motAB*, *cheAWMRBYZ*, *flhBAE*, *flgLKLIHGFEDCBAMN*	Chromosome 1'987'639-1'976'661, 1'998'119-2'005'128, 2'025'740-2'041'357, 2'847'590-2'859'159 (CP041031)
ETT2 locus (degenerate)	Type III secretion system (28)	EPEC, STEC	*eivACI*, *eivJ12*, *epaOPQR*, *epaS12*, *eivH*, *eprHIJK*	Chromosome 951'137-968'978 (CP041031)
Yersiniabactin	Siderophore (13)	ExPEC, UPEC	*fyuA*, *irp1*, *ipr2*, *ybtAEPQSTUX*	Chromosome 1'904'355-1'933'185 (CP041031)
Enterobactin	Siderophore (13)	EPEC, ExPEC and commensals	*entABCDEFH*, *entS*, *fepABCDEG*, *fes*, *ybdz*	Chromosome 3'404'020-3'423'654 (CP041031)
Agn43	Autotransporter (13)	ExPEC, UPEC	*flu*	Chromosome 52'072-49'228 (CP041031)
Aida-like protein	Autotransporter	Yet unspecified	Similar to *ehaB*	Chromosome 3'615'037-3'617'682 (CP041031)
Colanic acid operon	Capsule production (12)	ExPEC, Yet unspecified	*wzabc*, *wcaABCDEFJKLM*, *gmd*, *fcl*, *wcaI*, *cpsBG*, *wzxC*	Chromosome 1'789'589-1'812'389 (CP041031)
Iron related receptor[a]	Iron transport	Yet unspecified	*tonB-like*	pLB_CTX-M-15 6'210-8'180 (CP041032)
Colicin A1 Immunity protein	Colicin tolerance	Yet unspecified	Yet unspecified	pLB_CTX-M-15 28'608-28'943 (CP041032)
CjrABC	Iron acquisition (14)	ExPEC, UPEC	*cjrABC*	pLB_CTX-M-15 31'413-35'502 (CP041032)
SenB	Toxin (14)	ExPEC, UPEC	*senB*	pLB_CTX-M-15 35'571-36'746 (CP041032)
Aerobactin	Siderophore (13)	UPEC, APEC	*iucABCD*, *iutA*	pLB_CTX-M-15 108'676-116'656 (CP041032)
Sit operon	Iron acquisition (13)	UPEC, APEC	*sitABCD*	pLB_CTX-M-15 119'979-123'428 (CP041032)
Arginine deiminase operon	Increased fitness (UTI) (15)	UPEC	*arcACBDR*	pLB_CTX-M-15 79'550-84'792 (CP041032)

[a]The protein derived from the gene is related to transferrin/lactoferrin family receptor
[b]Groups of pathogenic *E. coli*, with which the virulence factors are the most commonly associated: AIEC, adherent-invasive *E. coli*; APEC, avian pathogenic *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EPEC, Enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; NMEC, neonatal meningitis-causing *E. coli*; SEPEC, sepsis-associated *E. coli*; UPEC, uropathogenic *E. coli*.

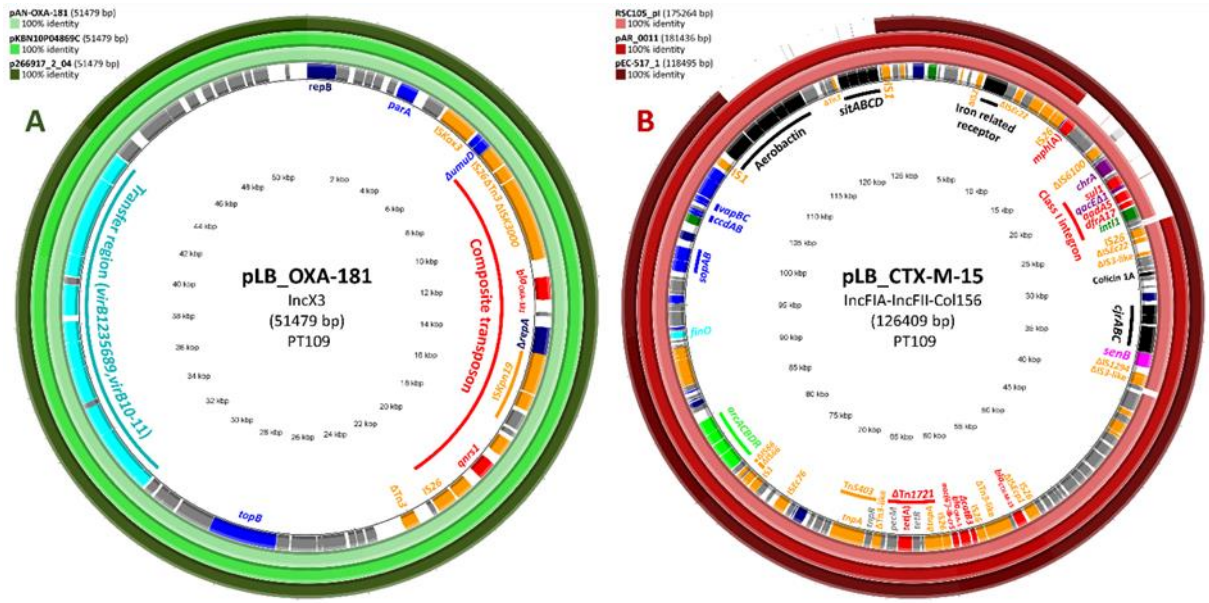

Figure 1. Circular map of plasmid pLB_OXA-181 (A, CP041033) and plasmid pLB_CTX-M-15 (B, CP041032) found in PT109.

The colored outer rings represent regions of homology that both plasmids share with their three closest relative plasmids found in the GenBank database. Genes present in pLB_OXA-181 (A) and pLB_CTX-M-15 (B) are portrayed as arrows in the inner ring of each circular map and are colored depending on their gene function classification. (A) First outer ring (light green), p_AN-OXA-181 (MK416154); second outer ring (green), pKBN10P04869C (CP026476); third outer ring (dark green), p266917_2_04 (CP026727). (B) First outer ring (pink), RSC105_pI (LO017737); second outer ring (red), pAR_0011 (CP024856); third outer ring (bordeaux), pEC517_1 (CP018964). The scale circle shows the coordinates in kilobase pairs of the reference plasmid. Antibiotic resistance genes and their functions: aadA, streptomycin/spectinomycin adenyltransferase; *aac(6')-Ib-cr*, aminoglycoside and quinolone acetyltransferase; *bla*$_{\text{OXA-1}}$, β-lactamase; *bla*$_{\text{CTX-M-15}}$, extended-spectrum β-lactamase; *bla*$_{\text{OXA-181}}$, carbapenemase; dfrA17, trimethoprim-insensitive dehydrofolate reductase; *mph*(A), macrolide phosphotransferase; *sul1*, sulfonamide-insensitive dihydropteroate synthase; *tet*(A), tetracycline efflux pump; *qnrS1*, DNA gyrase protection gene conferring low level resistance to fluoroquinolones. Genes are presented by colored arrows: red, antibiotic resistance genes; lime, genes associated with higher fitness; purple, genes associated with resistance to disinfectants and heavy metals; orange, transposase gene; green, integrase genes; light turquoise, conjugation associated genes; blue, genes associated with partition,modification and stability systems; dark blue, replication genes; black, virulence genes; pink, toxin genes; grey, other genes.

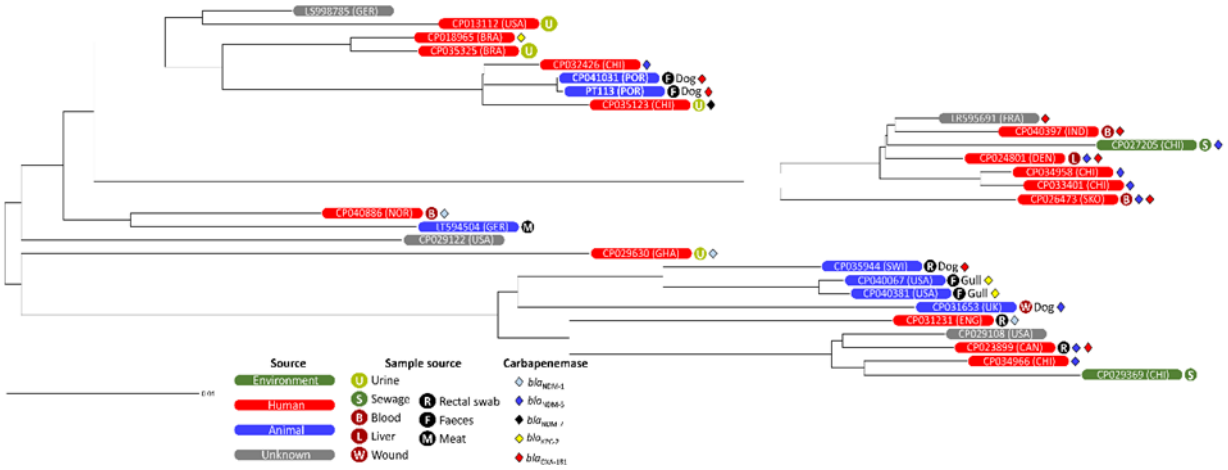

Figure 2. Phylogenetic neighbor-joining tree of all complete and/or circular genomes of *Escherichia coli* ST410 strains available in the GenBank database (n=26; accessed August 5th, 2019) together with PT109 (GenBank acc. no. CP041031) and PT113.

The phylogenetic neighbor-joining tree was based on the parameters “pairwise ignoring missing values” and “% columns difference”. The accession numbers of the sequences included in the tree are shown in the rounded rectangles, which are colored depending on their isolation source, and when provided, detail of the isolation source and carbapenemase production are shown by colored circles and rectangles, respectively (displayed by the legend). Accession number used were LS998785 (strain EC-TO75), CP013112 (YD786), CP018965 (Ecol_517), CP035325 (BR12-DEC), CP032426 (SCEC020001), CP041031 (PT109), CP035123 (EC25), LR595691 (EcMAD1), CP040397 (BA22372), CP027205 (WCHEC025943), CP024801 (AMA1167), CP034958 (SCEC020026), CP033401 (WCHEC020031), CP026473 (KBN10P04869), CP040886 (K71-77), LT594504 (RL465), CP029122 (AR434), CP029630 (ST410), CP035944 (AR24.2b), CP040067 (A1_181), CP040381 (A1_180), CP031653 (UK_Dog_Liverpool), CP031231 (Es_ST410_NW1_NDM_09_2017), CP029108 (AR437), CP023899 (FDAARGOS_433), CP034966 (WCHEC020032) and CP029369 (WCHEC035148).

Supplementary Table S1. Distribution of antibiotic resistance genes (ARGs) from E. coli PT109 and PT113 and the complete and circular genomes of ST410 E. coli from GenBank (n=26, accessed August 5th, 2019)

Antibiotic class	Antibiotic resistance genes[a]	Strains																											
		P109/PT113	A1_180	A1_181	AMA1167	AR24.2b	AR434	AR437	BA22372	BR12-DEC	EC25	ecmad1	Ecol_517	EC-TO75	Es_ST410_NW1_NDM_09_201	FDAARGOS_433	K71-77	KBN10P04869	RL465	SCEC020001	SCEC020026	ST410	UK_Dog_Liverpool	WCHEC020031	WCHEC020032	WCHEC025943	WCHEC035148	YD786
Aminoglycosides	*aac(3)-IId*				■		■		■		■	■					■	■			■			■	■	■		
	aac(3)-IV																									■		
	aac(6')-Ib-cr	■			■		■	■	■		■	■	■			■	■	■		■	■	■		■		■		
	aac(6')-Ib3						■										■											
	aac(6')-Ic		■																									
	aadA1																		■			■						■
	aadA2		■		■											■		■				■	■					■
	aadA5	■		■	■				■	■	■	■	■		■	■		■			■			■	■			
	aadA16										■	■														■		
	aadA22																									■		
	aph(3')-Ia			■															■							■		
	aph(3'')-Ib		■	■	■				■		■	■			■			■	■		■			■		■		■
	aph(6)-Id		■	■	■				■		■	■			■			■	■		■			■		■		■
	armA														■													
	rmtB															■										■		
	rmtC																■											
Beta-lactams	*bla*CMY-2				■				■			■						■			■			■		■		
	*bla*CMY-6																■											
	*bla*CMY-42					■										■							■		■		■		
	*bla*CTX-M-2																											■
	*bla*CTX-M-3										■				■										■			
	*bla*CTX-M-15	■			■			■	■			■	■	■		■		■	■		■	■						
	*bla*CTX-M-65																									■		
	*bla*KPC-2		■	■									■															
	*bla*NDM-1														■		■					■						
	*bla*NDM-5				■											■		■		■	■		■	■	■	■		
	*bla*NDM-7										■																	
	*bla*OXA-1	■			■			■	■		■	■	■			■	■	■		■	■			■				
	*bla*OXA-9			■																		■						
	*bla*OXA-181	■			■	■			■			■				■		■										
	*bla*SHV-12		■																									
	*bla*TEM-1			■	■				■		■	■				■		■	■		■	■		■		■		
	*bla*TEM-176																											■
	*bla*TEM-190																						■					
	*bla*VEB-5						■																					
Colistin	*mcr-1.1*																		■							■		
	mcr-3.5																									■		
Fosfomycin	*fosA*																											■
Macrolides	*erm*(B)			■																					■			
	erm(42)		■	■																								
	msr(E)			■											■													
	lnu(F)																									■		
Phenicol	*cmlA1*																					■						
	floR		■	■																						■		■
Quinolones	*qnrB2*		■								■																	
	qnrB52										■																	
	qnrS1	■			■	■			■		■	■				■		■									■	
	qepA1														■													
	oqxAB																									■		
Rifampicin	*arr-3*										■	■														■		
Sulfonamides	*sul1*		■	■	■		■		■	■	■	■	■		■	■	■	■			■		■	■	■	■		■
	sul2		■	■	■				■		■	■			■			■	■		■			■		■		
	sul3																		■			■						■
Tetracyclines	*tet*(A)	■	■	■				■		■	■		■		■	■			■	■						■	■	
	tet(B)				■		■		■			■						■			■			■				
	tet(M)																									■		
Trimethoprim	*dfrA1*																		■									
	dfrA8							■																				
	dfrA12				■											■		■				■	■					■
	dfrA14													■												■	■	
	dfrA17	■		■	■				■	■	■	■	■		■			■			■			■	■			
	dfrA19		■																									
	dfrA27										■	■														■		

Supplementary Table S2. Distribution of virulence factors from OXA-181-producing E. coli PT109 and PT113 shared with other circular and complete genomes of ST410 strains from GenBank (n=26, accessed August 5th, 2019)

Virulence factor combinations (number of strains)	Strains (accession numbers) sharing virulence factors of PT109/PT113
Common twelve virulence factors shared among all ST410 strains from NCBI (n=26) included in this study: Hemorrhagic *E. coli* pilus CFA/I fimbriae Intimin-like FdeC *E. coli* common pilus Type 1 fimbriae Stg fimbriae UpaG adhesin *E. coli* laminin-binding fimbriae NlpI lipoprotein Flagella Aida-like protein Enterobactin	A1_180 (CP040381-P040389) A1_181 (CP040067- P040076) AMA1167 (CP024801-CP024806) AR24.2b (CP035944; MK416154-MK416155) AR434 (CP029122- CP029123) AR437 (CP029103-CP029108) BA22372 (CP040397-CP040399) BR12-DEC (CP035325-CP035329) EC25 (CP035123-CP035123) EcMAD1 (LR595691-LR595694) Ecol_517 (CP018963-CP018965) EC-TO75 (LS998785-LS998788) Es_ST410_NW1_NDM_09_2017 (CP031231-CP031235) FDAARGOS_433 (CP023895-CP023905) K71-77 (CP040884-CP040886) KBN10P04869 (CP026473-CP026476) RL465 (LT906556-LT906558; LT594504) SCEC020001 (CP032420-CP032426) SCEC020026 (CP034954-CP034958) ST410 (CP029630-CP029631) UK_Dog_Liverpool (CP031653-CP031658) WCHEC020031 (CP033397-CP033401) WCHEC020032 (CP034959-CP034966) WCHEC025943 (CP027199-CP027205) WCHEC035148 (CP029365-CP029369) YD786 (CP013112, KU254578-KU254581)
Common+Curli fibers (n=25)	All 26 strains included in the study except AR24.2b.
Common+F9 fimbriae (n=24)	All 26 strains included in the study except AR24.2b and K71-77.
Common+ETT2 degenerate locus (n=24)	All 26 strains included in the study except RL465, BR12-DEC.
Common+Agn43 (n=19)	All 26 strains included in the study except AR24.2b, AR434, ST410, WCHEC020032, AR437, FDAARGOS_433, WCHEC035148.
Common+Sit operon (n=11)	RL465, AR437, FDAARGOS_433, WCHEC035148, K71-77, UK_Dog_Liverpool, Es_ST410_NW1_NDM_09_2017, BR12-DEC, Ec-TO75, EC25, SCEC020001.
Common+Iron related receptor[a] (n=10)	AR437, FDAARGOS_433, WCHEC035148, K71-77, UK_Dog_Liverpool, BR12-DEC, Ec-TO75, EC25, SCEC020001 and Ecol_517.
Common+Aerobactin (n=10)	AR437, FDAARGOS_433, WCHEC035148, Es_ST410_NW1_NDM_09_2017, K71-77, UK_Dog_Liverpool, BR12-DEC, Ec-TO75, EC25, SCEC020001.
Common+Colonic acid operon (n=7)	KBN10P04869, YD786, BR12-DEC, Ec-TO75, EC25, SCEC020001 and Ecol_517.
Common+Yersiniabactin (n=6)	KBN10P04869, BR12-DEC, Ec-TO75, EC25, SCEC020001 and Ecol_517.
Common+Colicin A1 Immunity protein (n=2)	BR12-DEC and Ecol_517.
Common+CjrABC (n=1)	Ecol_517.
Common+SenB (n=1)	Ecol_517.

Chapter 5

Discussion, conclusions and future perspectives

5.1 General discussion

E. coli are Gram-negative bacteria that are ubiquitous colonizers of the gastro-intestinal tract in humans and companion animals. *E. coli* is commonly isolated from clinical samples of companion animals with infections of the urinary, respiratory, skin and soft tissue and gastro-intestinal tract (Guardabassi et al. 2004; Pomba et al. 2017; Zogg et al. 2018). Over the last years, ESBL and carbapenemase-producing *E. coli* have been isolated, with increasing frequency, from humans and from healthy and diseased companion animals (Coque et al. 2008; Ewers et al. 2012; Dolejska et al. 2013; Gandolfi-Decristophoris et al. 2013; Belas et al. 2014; Baede et al. 2015; Bogaerts et al. 2015; Melo et al. 2017; Grontal et al. 2018; Hong et al. 2018; Nigg et al. 2019; Reynolds et al. 2019; Sheu et al. 2019). Carbapenemase-resistant and ESBL-producing Enterobacterales, such *E. coli,* are at the top of the WHO priority list (WHO 2019). ESBL/carbapenemase genes are carried on plasmids, often by bacteria belonging to clones with properties that facilitate its transmission. However, the clinical significance of these isolates and their involvement in animal disease, occurring as either opportunistic pathogens or simple colonizers, has been very rarely investigated. Futhermore, there is an important gap of knowledge concerning the link between cephalosporin usage, emergence of antimicrobial resistance and the potential transmission of ESBLs/carbapenemase-producing *E. coli* by direct human-companion animal contact.

5.1.1 ESBLs/ carbapenemase - producing Extraintestinal Pathogenic *Escherichia coli* in companion animals and humans without direct contact

Chapter 3.1 describes the first report of the disseminated *E. coli* O25b:H4-B2-ST131-H30/H30-Rx, MDR, fluoroquinolone-resistant human high-risk clone and its CTX-M-15-*H*30-Rx and CTX-M-1-*H*30-Rx subsets in companion animals with UTI from Europe. Between 1999 and 2015, 342 uropathogenic *E. coli* were isolated at the Laboratory of Antibiotic Resistance, Faculty of Veterinary Medicine, University of Lisbon from companion animals with UTI. Seven of the O25b:H4-B2-ST131 clone companion animal isolates were the *H*30 subclone and three out these seven were the *H*30-Rx subclone. Furthermore, all *H*30/*H*30-Rx subclones causing UTI in

companion animals belonged to virotype D, which confirms their virulent characteristics (Nicolas-Chanoine et al. 2014). Moreover, one isolate carried the ESBL *bla*$_{CTX-M-1}$ gene, which is associated with *E. coli* isolates of farm animal origin and described in humans in Turkey with UTI (Nicolas-Chanoine et al. 2014; Can et al. 2016). Furthermore, one O25b:H4-B2-ST131-*H*30 isolate harboured both *oqxA* and *oqxB* efflux pump genes, which could be potentially related to its reduced fluoquinolone susceptibility. These results raise public health concerns (Banerjee et al. 2013) since these subclones may have an impact on human health through the close and direct contact of companion animals with their owners. Moreover, the close contact between companion animals and humans creates opportunities for interspecies transmission of resistant bacteria. Studies of ST131 *H*30/*H*30-Rx in humans in Portugal are scarce (Rodrigues et al. 2016; Belas et al. 2019). However, *H*30/*H*30-Rx subclones have been previously described in faecal samples from healthy humans (Rodrigues et al. 2016).

Chapter 3.2 aimed to characterize ESBL/AmpC ß-lactamase producing - *E. coli* strains causing UTI in companion animals and non-related humans from the community. Thirty-five non-duplicate third – generation cephalosporins resistant - *E. coli* isolates causing UTI in companion animals were studied and compared to 85 non-duplicate third – generation cephalosporins resistant - *E. coli* isolated from non-related humans with community-acquired UTI.

In this chapter a large diversity of ESBL/AmpC ß-lactamase producing *E. coli* from companion animals and non-related humans with community-acquired UTI were identified. However, in companion animals, cephalosporin resistance was frequently associated with the presence of *bla*$_{CTX-M-15}$ and *bla*$_{CMY-2}$ genes, while in humans with community-acquired UTI it was associated with *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-1}$. Furthermore, the frequency of CMY-2-producing *E. coli* strains in companion animals was higher than in humans with community-acquired UTI. In a study performed in UK, the authors also found a high prevalence of CMY-2- producing *E. coli* strains from dogs with UTI (Wagner et al. 2014). Most CMY-2-producing *E. coli* strains belonged to the phylogenetic group D, which is consistent with a previous study performed in the United States (Liu et al. 2016). The majority of third-generation cephalosporins-resistant *E. coli* strains belonging to the phylogenetic group B2 and had a high number of the pathogenicity island markers. The association of group B2 and several pathogenicity island markers has previously been reported among uropathogenic *E. coli* strains (Sabaté et al. 2006). Furthermore, the A, B1, and D phylogenetic groups of uropathogenic *E. coli* strains from the present study also have less pathogenicity island markers as described before by other authors (Sabaté et al. 2006; Mateus et al. 2013).

In this chapter, two multidrug-resistant high-risk clonal lineages, the ST131 and ST648, and also the ST88 were shared between companion animals and humans with UTI. *E. coli* ST88 clone has been associated with poultry and broiler meat, which may suggest that animals from

farms are reservoirs for this type of *E. coli* that is able to cause extraintestinal disease in humans and companion animals (Day et al. 2016; van Hoek et al. 2018; Borges et al. 2019).

bla$_{CTX-M-15}$ and *bla*$_{CTX-M-14}$ genes are the most common types of ESBLs among strains isolated from human with UTI, though *bla*$_{CTX-M-14}$ gene has mainly been reported in Japan, Asian countries, Canada and Spain (Peirano et al. 2010; Zong and Hu 2013).

The ST131 clone harbouring *bla*$_{CTX-M-15}$ or *bla*$_{CTX-M-14}$ has also been detected among companion animals in many countries (Ewers et al. 2010; Pomba et al. 2014; Belas et al. 2019). In Japan, between 2005 and 2010, Matsumura et al. (2016) found the *bla*$_{CTX-M-14}$ gene to be the most common, followed by *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-2}$. They also recently reported the global emergence and increased prevalence of the *E. coli* ST131 sub-clade (C1/*H*30R1, named the C1-M27). The *bla*$_{CTX-M-27}$, a single-nucleotide variant of *bla*$_{CTX-M-14,}$ has been increasingly identified among the *E. coli* strains from humans and companion animals with UTI in the United States and in Asian and European countries (Harada et al. 2012; Matsumura et al. 2016; Bevan et al. 2017). It has also been reported in clinical *E. coli* strains from companion animals from France (Melo et al. 2019). Moreover, the transmission of the *E. coli* ST131 clone among family members and companion animals has been documented (Johnson et al. 2009a).

The results of this chapter suggest that the C2/*H*30Rx subclade is a prevalent clone in humans with community-acquired UTI from the Lisbon area, and that the majority of these isolates harbour a small number of ESBL-gene types. This clone has previously been demonstrated as being highly virulent and, when multidrug-resistant, it may have a direct impact in the management of community-associated UTI in both humans and companion animals. This clone is normally associated with complicated UTIs (Campos et al. 2018). To the best of our knowledge, in this study we have the first description of *E. coli* O25b:H4-ST131 harbouring *bla*$_{CMY-2}$ gene and also, the first description of the subclade C1-M27 in humans with community-acquired UTI in Portugal. However, C1-M27 subclade has been detected in faecal samples of healthy humans in the north of Portugal (Rodrigues et al. 2016). Moreover, *E. coli* O25b:H4-ST131 harbouring *bla*$_{CMY-2}$ gene has been rarely described (Day et al. 2016; Hansen et al. 2016).

Previous studies of clinical *E. coli* showed that the O16-H5-ST131 clone (clade A) is globally distributed (Johnson et al. 2014). In this thesis we have, to best of our knowledge, the first description of the O16:H5-ST131 clone harbouring *bla*$_{CTX-9like}$ in a human with community-acquired UTI in Portugal. Moreover, it is noteworthy that ST648 strains were strongly associated with *bla*$_{CMY-2}$ gene, combining both multidrug-resistant and virulent phenotypes. Further studies about ST648 strains in companion animals are needed, to clarify if there are possible routes of the transmission of this clone to humans by direct contact or by environmental contamination.

These findings are of critical relevance, as they show the role of companion animals and humans as reservoirs of pandemic clones, especially *E. coli* ST131-C2/*H*30Rx (*bla*$_{CTXM-15}$) and ST648 harbouring CMY-2. Furthermore, *E. coli* ST10 and ST410, other important pandemic

lineages, were among the STs detected in humans with community - acquired UTI (Mathers et al. 2015; Campos et al. 2018). Yet, these ST types were not detected in companion animals with UTI in Lisbon.

This chapter allows to understand some aspects of the dissemination of ESBLs/AmpC-producing *E. coli* in the Lisbon area, which is an important step for developing strategies to prevent the propagation of high-risk clones. In a One-Health perspective, the collaboration between Veterinary medicine and Human medicine is needed to characterize the occurrence and routes of dissemination of these high-risk clones. Considering that companion animals with UTI are generally treated at home by the owners, measures should be implemented to avoid the spread of these bacteria to the environment.

In chapter 3.3, the purpose of the study were to evaluate the presence and load of ESBL/AmpC-producing Enterobacteriaceae faecal carriage in healthy dogs after elective surgery and to identify potential risks factors associated with faecal colonization. Faecal samples were collected from dogs submitted to surgical procedures (n=25). Faecal samples from the surgery group were collected before surgery and after surgery. Interestingly, about 20% of dogs before surgery and before entering the hospital were already colonized with ESBL/AmpC-producing Enterobacterales. The results obtained in this study were quite similar to the results obtained previously (Belas et al. 2014). ESBL/AmpC-producing Enterobacterales implicated in this study were *E. coli*, *K. pneumoniae* and *E. cloacae.* Furthermore, the bla_{TEM} and $bla_{\text{CTX-M-1 group}}$ genes were the most frequent β-lactam-resistance genes detected, which is in agreement with previous studies (Costa et al. 2007; Belas et al. 2014). Hordijk (2013), in the Netherlands, analysed healthy dogs and cats without contact with the hospital environment and detected a high percentage (45%) of dogs colonized with Enterobacterales producing β-lactamases (ESBL/AmpCs). Procter (2014) reported that 12.7% of *E. coli* strains isolated from dogs who attended parks in three cities from Canada, were resistant to β-lactam antimicrobials. The different frequencies of β-lactam resistant bacteria obtained in these studies may be linked to the different geographical regions or simply be due to differences in the bacteria isolation methodology used. The frequency of ESBL-producing Enterobacterales significantly increased during antimicrobial administration and changes in fecal microbiota occurred. These results could be in part explained by the use of amoxicillin-clavulanate in small animal practice. The use of β-lactams has been previously associated with an increased risk of carriage of antimicrobial resistant *E. coli* in dogs (Rantala et al. 2004; Ogeer-Gyles et al. 2006; Belas et al. 2014). These results are extremely important, emphasizing the need for appropriate antimicrobial prophylaxis guidelines. This issue is of great importance not only because of the direct impact on patients, but also because resistant bacteria can be transmitted from companion animals to humans (Johnson et al. 2001, Guardabassi et al. 2004; Johnson et al. 2009; Pomba et al. 2017).

ESBL/AmpC-producing Enterobacterales may also spread from patient-to-patient due to inadequate attention to infection control measures, especially hand washing. Infections caused by Enterobacterales have features that are of particular concern. These organisms are highly efficient at up-regulating or acquiring genes that code for mechanisms of antimicrobial drug resistance, especially in the presence of antimicrobial selective pressure (Peleg and Hopper 2010). Furthermore, nowadays, there is evidence that the composition of the gut microbiota may change in response to external factors such as antimicrobials and environment (Coyte et al. 2015). Antimicrobial administration creates alterations in the faecal microbiome and can affect the immune system and the health of the host (Ubeda et al. 2012; Panda et al. 2014).

Antimicrobials must be used responsibly and restrictively to minimize resistance, retain the efficacy of the currently available antimicrobial agents, and to maintain a healthy gut microbiome (Grønvold et al. 2010).

5.1.2 ESBLs/ carbapenemase - producing Extraintestinal pathogenic *Escherichia coli* in companion animals and humans with direct contact

Chapter 4.1 reports the sharing of clinically important antimicrobial resistance genes by companion animals and their human household members. The aims of this chapter were to implement a rapid easy methodology, to characterize the antimicrobial resistant gene gut content associated with Enterobacterales and staphylococci; and to evaluate statistical associations between antimicrobial resistance genes present in fecal samples from healthy companion animals and their human household members.

Fecal samples were collected from humans (*n*=27) and companion animals (*n*=29) living in close contact in 20 households. Furthermore, healthy humans without daily contact with companion animals (*n*=19) were enrolled to the control group.

The antimicrobial resistance genes studied are responsible for phenotypic resistance to six antimicrobial classes: β-lactams, aminoglycosides, colistin, trimethoprim/sulfamethoxazole, tetracycline and chloramphenicol in Enterobacterales and in *Staphylococcus* spp.

Nowadays, companion animals live in a “relationship of mutualism” with their owners (Dotson and Hyatt 2008). The anthropomorphizing of companion animals has led to changes in the behavior of owners towards them, with increasing conducts like kissing, licking, sharing food and sharing beds. Considering the shared environment of humans and companion animals, their close relationship, and the increased frequency of antimicrobial-resistant bacteria detected in humans and companion animals, new opportunities are created for interspecies transfer of antimicrobial resistance genes (Dotson and Hyatt 2008; Pomba et al. 2017).

Antimicrobials are used extensively in human medicine, veterinary medicine, food-producing animals and agriculture (Rolain 2013). In Portugal, β-lactams, such as penicillins, are

the most prescribed antimicrobials in humans followed by macrolides, lincosamides, streptogramins, quinolones, tetracyclines and sulfonamides-trimethoprim (ECDC 2018). In Veterinary medicine, penicillins are also the most commonly prescribed antimicrobials in companion animals (EMA 2018). Yet, lincosamides, quinolones, macrolides, tetracyclines, nitroimidazoles and sulfonamides-trimethoprim are also used in small animal practice (EMA 2015; EMA 2018). Several antimicrobial classes that are used in humans and companion animals are the same, leading to an overlap of the detected antimicrobial resistance genes (Guardabassi et al. 2004; Pomba et al. 2017). Enterobacterales resistance to β-lactams is increasing in humans and in companion animals and there are no specific β-lactamases that are restricted only to animals or humans (Schnellmann et al 2006). This seems to be in line with the results from this study. The β-lactamases that are disseminated in the Enterobacterales family, especially the ESBLs and cephalosporinases of AmpC type are of particular clinical relevance.

In this chapter, the *bla*$_{\text{TEM}}$ gene was the most frequent β-lactam-resistance gene in humans and companion animals, which is in agreement with previous studies (Trott 2012; Costa et al. 2007; Rodríguez-Baño et al. 2008). In Portugal, the TEM-β-lactamase has also been detected in Enterobacterales from food-producing animals and from commensal and clinical isolates (Pomba-Féria and Caniça 2003; Costa et al. 2007). The *bla*$_{\text{TEM}}$ genes detected in this study (from companion animals and humans) had a similar promotor and coding region polymorphisms as the *bla*$_{\text{TEM-1B}}$ (according to the Sutcliffe numbering system) (Sutcliffe 1978). This finding may have resulted from a zoonotic transfer of *bla*$_{\text{TEM-1B}}$ genes harboured in Enterobacterales. Nevertheless, a common source of colonization could also be hypothesized since this resistance mechanism has been extensively detected in Portugal (Pomba-Féria and Caniça 2003; Costa et al. 2007).

CTX-M β-lactamases are the current dominant type of ESBLs worldwide, having overpassed the TEM and SHV β-lactamases in Europe, both in humans and animals (Ewers et al. 2011; Belas et al. 2014). Furthermore, Portugal is among the European countries with the highest frequency of ESBL detection, mainly TEM and CTX-M (Machado et al. 2013; Fernandes et al. 2014). However, in this chaper, only the ESBL SHV-27 gene was detected in a healthy human from the control group. The *bla*$_{\text{SHV-27}}$ gene has been previously detected in clinical *K. pneumoniae, E. coli* and *Enterobacter cloacae* from humans and in clinical *K. pneumoniae* from dogs of different countries (Abbassi et al. 2008; Kiratisin et al. 2008; Hammami et al. 2011; Zhang et al. 2018). To the best of our knowledge, this is the first detection of *bla*$_{\text{SHV-27}}$ gene in fecal samples from healthy humans in Portugal and in Europe.

Only the CMY-2 and DHA-1 beta-lactamase encoding genes were detected among all the AmpC cephalosporinase genes tested in this study, and these occurred mainly in humans. The detection of these AmpC genes was already described in Portugal in clinical strains of

Enterobacterales from humans in the community and hospital, but as far as we are aware, not in healthy individuals in the community (Ribeiro et al. 2019).

The effect of the low antimicrobial consumption, the household controlled environment, and the possible food-borne dissemination of antimicrobial resistance genes should also be considered in this study as an explanation for the shared antimicrobial resistance genes (Pitout et al. 2005; Gong et al. 2018; Zhang et al. 2018, Zhang et al. 2019). The presence of ESBLs/AmpC in this chapter was lower than previously reported in healthy dogs in Portugal (Lisbon area) (Belas et al. 2014). In a previous study, dogs from shelters/breeders were approximately three times more likely to have an ESBL/AmpC-producing *E. coli* than dogs from private owners (Belas et al. 2014). The results in the present study may be explained by the fact that the companion animals included in this study had little contact with kennels and were healthy.

This chapter 4.1 showed that humans and companion animals carried and shared several antimicrobial resistance genes of clinical importance. Most of these genes are usually associated with mobile genetics elements, which are important for the antimicrobial resistance transfer between different microbiomes (Karami et al. 2006; Kumer et al. 2004; Broaders et al 2013; Jernberg et al. 2013; Gillings et al. 2014; Cornican et al. 2017). The role of companion animals in the dissemination of clinically relevant antimicrobial resistance genes to humans through fecal contamination should not be neglected. Further studies are needed to determine the causality and directionality of resistance genes transfer between human and companion animals, in order to identify the critical control points at which interventions could substantially prevent the spread of antimicrobial resistance genes within households and establish the prevention and intervening measures for controlling resistance.

The chapter 4.2 reports two multidrug-resistant and carbapenemase-producing *E. coli* clones of ST410 isolated from fecal samples of a dog with skin infection at admission to an animal hospital in Portugal, and one month after discharge. Whole-genome sequencing revealed a 126,409-bp Col156/IncFIA/IncFII multidrug-resistance plasmid and a 51,479-bp IncX3 $bla_{\text{OXA-181}}$-containing plasmid. The chromosome and plasmids carried virulence genes characteristic of uropathogenic *E. coli* indicating that dogs may carry multidrug-resistant *E. coli* related to those causing UTI in humans.

Fecal samples of 71 healthy companion animals (n=47 dogs and n=24 cats) were collected at home, whilst those of companion animals with UTIs (n=13 dogs and n=2 cats) and 12 companion animals with skin and soft-tissues infections (n=11 dogs and n=1 cat) were taken at admission to the University teaching hospital for carbapenemase-producing bacteria screening. This chapter provided an in-depth characterization of the first OXA-181-producing Extraintestinal Pathogenic *E. coli* obtained in a veterinary environment and its comparison with other ST410 strains. Detection of the same clone within a 1-month period indicates that such a multidrug-resistant and carbapenemase-producing pathogenic *E. coli* can temporarily persist in

dogs and disseminate into the environment, other animals and humans, therefore posing a major One Health concern (Pulss et al, 2018; Pitout et al. 2019).

5.2 Conclusions

The results obtained in theses studies on the transmission of ESBLs/pAmpC and carbapenemases-producing Extraintestinal Pathogenic *E. coli* contributed with important and updated epidemiological information to small animal veterinary medicine and public health.

5.2.1 ESBLs/ carbapenemase - producing Extraintestinal Pathogenic *Escherichia coli* in companion animals and humans without direct contact

To our best knowledge the high-risk clonal lineages *E. coli* O25b:H4-ST131-*H*30/*H*30Rx were here described for the first time in companion animals (dogs and cats) from Portugal and Europe. Moreover, these subclones belonged to the virotype D, which confirms their patogenicity and virulent characteristics.

The detection of high-risk clonal lineages harbouring clinically relevant antimicrobial-resistant mechanisms, such as third – generation cephalosporins *E. coli* O25b:H4-B2-ST131, CC23 and ST648, highlights the role of companion animals with UTI in its dissemination.

The bla_{CMY-2} producing *E. coli* ST648 is the most common high-risk clonal lineage causing UTI in companion animals from Lisbon area. The cross-species sharing of important multidrug-resistant high-risk clones is a public health concern. Furthermore, these high-risk clonal lineages pose a therapeutic dilemma given that they often are only susceptible to antimicrobials critically important for humans.

The detection and of faecal pathogenic ESBLs/ AmpC-producing *E. coli* in healthy dogs is also an important finding, because resistant bacteria can be transmitted from animal to animal and to humans.

It is important to implement some measures to avoid the spread of these bacteria to the environment.

5.2.2 ESBLs/ carbapenemase - producing Extraintestinal Pathogenic *E. coli* in companion animals and humans with direct contact

To our best knowledge, the possibility for sharing of clinically important antimicrobial resistance genes by companion animals (dogs and cats) and their human household members was demonstrated for the first time. Companion animals also seem to be reservoirs of clinically important resistance genes, such β-lactams genes (classe A and C) which supports their role as reservoirs.

The detection of faecal high-risk clone OXA-181 producing- *E. coli* ST410 strains that was closely related to uropathogenic clinical human strains is also an important finding and to the our best knowledge was here first described in Portugal and Europe. Considering that this pandemic lineage is involved in the spread of ESBLs and carbapenemases in humans, the role of companion animals in its spread should not be neglected and raises great public-health concerns.

5.3 Future perspectives

The studies presented point to some research that needs to be further explored in the future.

5.3.1 ESBLs/ carbapenemase - producing Extraintestinal Pathogenic *Escherichia coli* in companion animals and humans without direct contact

Future studies using whole genome sequencing (WGS) are needed to link the population structure and molecular antimicrobial resistance epidemiology of Extraintestinal pathogenic *E. coli* of companion animal and human origins. Moreover, E. coli from skin and soft tissue infections need to be studied to understant which types of infection (SSTI or UTI) promote a higher risk of transmission. Also, studies are needed to investigate similiarities between Extraintestinal pathogenic *E. coli* from companion animals and humans and to assess possible risk factors.

5.3.2 ESBLs/ carbapenemase - producing Extraintestinal Pathogenic *Escherichia coli* in companion animals and humans with direct contact

In this thesis it was demonstrated that companion animals and humans may share clinically important antimicrobial resistant genes and high - risk clones. Therefore, more studies are needed to clarify the transfer of antimicrobial resistance genes/antimicrobial-resistant *E. coli* from companion animals to humans and vice-versa. Moreover, the use of a longitudinal design would benefit the clarification of the colonizaion and transmission dynamics over time. The study of a bigger sample size will allow the identification of possible risk factors and transmission routes, which in turn will allow the determination of control and preventive measures to decrease the dissemination of ESBL/pAmpC/carbapenemases-producing and/or virulent *E.* coli belonging to high-risk clones.

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Annexes

During the phD, two book chapers (Annex 1 and 2) were published in Advances in Animal Health, Medicine and Production: A Research Portrait of the Centre for Interdisciplinary research in Animal Health (CIISA), University of Lisbon, Portugal. Springer Nature Switzerland AG 2020. The chapters were about antimicrobial resistance and virulence in companion animals with UTI and about the public health risk of antimicrobial resistance transmission dynamics (animal- to-human and vice-versa) during different types of companion animals infections.

Annex 1 - Antimicrobial resistance trends in dogs and cats with urinary tract infection

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Antimicrobial resistance trends in dogs and cats with urinary tract infection

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Abstract

Urinary tract infections (UTI) are among the most common infections diagnosed in companion animals and usually require antimicrobial use. The antimicrobials available nowadays for UTI treatment are limited and most of them are considered as critically important to humans by the World Health Organization (WHO). Therefore, awareness of the antimicrobial resistance trends and underlying resistance mechanisms in uropathogenic bacteria from dogs and cats is of importance in a one health perspective.

This has long been one of the main research fields of the Antibiotic Resistance Laboratory Team. A recent and pivotal study from our team has given, for the first time, an European overview of the geographic distribution of antimicrobial resistance in uropathogenic bacteria from dogs and cats. The southern European countries, including Portugal, had significantly higher resistance frequencies than Northern countries, mirroring what happens in human invasive bacteria. Noteworthy, increasing antimicrobial resistance trends over 16 years were also detected in bacteria from companion animals with UTI.

The detection of multidrug resistant (MDR) extended spectrum (ESBLs)/ AmpC beta-lactamases - producing bacteria in companion animals with UTI is worrisome due to its clinical implications. Furthermore, companion animals UTI may frequently be caused by high-risk clonal lineages to humans like *Escherichia coli* ST131, ST648 and *Klebsiella pneumoniae* ST15 which underlines their public-health relevance.

Keywords: Antimicrobial resistance, animal infection, public health risk.

Urinary tract infections

Urinary tract infections (UTI) are among the most frequently diagnosed infections in companion animals (Weese *et al*., 2011). Is has been estimated that close to 14% of dogs visiting a veterinarian will develop a UTI during their lifetime (Thompson *et al.*, 2011). The frequency of urinary tract infections in cats with lower urinary tract disease is considered to be less than 2% (Gunn-Moore, 2003); however, some studies have found higher frequencies, varying between 8% and 25% (Lekcharoensuk *et al*., 2001; Gerber *et al*. 2005; Eggertsdóttir *et al.*, 2007; Sævik *et al.*, 2011). Therefore, UTIs are an important reason for the need to prescribe antimicrobials in small animal veterinary medicine. Considering its relevance, the study of UTIs and antimicrobial resistance in companion animals has been one major research field of the Antibiotic Resistant Laboratory in the last decades (Féria *et al*., 2000; Féria *et al*., 2002; Caniça *et al*., 2004; Pomba *et al*., 2009; Pomba *et al*., 2010; Mateus *et al*., 2013; Oliveira *et al*., 2014; Pomba *et al*., 2014a; Pomba *et a*l., 2014b; Marques *et al*., 2016; Marques *et al*., 2018a; Marques *et al*., 2018b; Belas *et al*., 2019; Marques *et al*., 2019).

Pathophysiology and predisposing factors

UTIs occur as a consequence of the failure of host defence mechanisms with subsequent adherence, multiplication and persistence of virulent bacteria in the urinary tract (Bartges, 2004). UTI are usually initiated by the adherence and colonisation of bacteria into the urethra followed by migration to the bladder. Successful bacteria will then multiply and colonise the bladder and eventually ascend to the kidney. Ultimately, bacteria will cross the tubular epithelial barrier into the blood stream, resulting in bacteraemia (Flores-Mireles *et al*., 2015).

There are several predisposing factors associated with higher frequencies of UTI including: *diabetes mellitus*, chronic kidney disease in cats, hyperthyroidism in cats, hyperadrenocorticism and bladder transitional cell carcinoma in dogs, anatomical abnormalities or diseases promoting urine retention and abnormal micturition, corticoid treatment (Saitoh *et al.*, 1985; Freshman *et al.*, 1989; Forrester *et al.*, 1999; Hess *et al*., 2000; Seguin *et al*., 2003; Torres *et al*., 2005; Bailiff *et al*., 2006; Stiffler *et al*., 2006; Graves *et al*., 2007; Mayer-Roenne *et al*., 2007; Bubenik and Hosgoof, 2008; Eriksson *et al.*, 2010; Hirji *et al.*, 2012; Martinez-Ruzafa *et al*., 2012; Budreckis *et al*., 2015). Furthermore, UTI is more frequent in female and older dogs and cats, spayed female dogs and Persian and Abyssinian cat's breeds (Lekcharoensuk *et al*., 2001; Ling *et al*., 2001; Cohn *et al*., 2003; Seguin *et al*., 2003; Bailiff *et al*., 2006; Graves *et al*., 2007; Mayer-Roenne *et al*., 2007; Bailiff *et al*., 2008).

Classification of urinary tract infections and diagnosis

In veterinary medicine, the Working Group of the International Society for Companion Animal Infectious Diseases (ISCAID) has developed a dedicated guideline for the antimicrobial treatment of UTI in companion animals (Weese *et al*., 2011). The first step in the UTI treatment decision making in this guideline relies on the classification of the type of UTI.

Bacteriuria can be detected in patients without the presence of clinical signs, then called asymptomatic bacteriuria (Weese *et al*., 2011). When present, clinical signs of UTI are not pathognomonic and include dysuria, pollakiuria, stranguria, haematuria, urgency to urinate, fever, abdominal/flank pain, vocalisation, among others (Bartges *et al*., 2004; Gerber *et al*., 2005; Weese *et al*., 2011; Passmore *et al.*, 2008).

According to the location, UTIs are considered upper UTIs or pyelonephritis (kidney) and lower UTIs or cystitis (bladder). Based on the frequency of UTI episodes within the last 12 months, UTIs are classified as simple (< 3 episodes) or recurrent (≥ 3 episodes) (Weese *et al*., 2011).

Cystitis are considered as uncomplicated when they are diagnosed in patients that are otherwise healthy (i.e. without comorbidities) and with normal genitourinary tract anatomy and function (Weese *et al*., 2011). These are always simple UTIs, since according to Weese *et al*. (2001) recurrence points to the presence of undiagnosed comorbidities. Complicated cystitis occurs in patients with comorbidities (e.g. urinary obstruction, renal failure and *diabetes mellitus*) or predisposing factors for UTI (Weese *et al*., 2011).

Diagnosis and classification of UTI requires the clinical evaluation of the patient, complete type II urinalysis, the necessary complementary diagnostic workout to diagnose suspected comorbidities and a urine culture (Weese *et al*., 2011). The presence of bacteriuria and pyuria in urine sediment strongly correlates with the presence of UTI, however it is not diagnostic (Bartges *et al*., 2004; Mayer-Roenne *et al.*, 2007).

Urine culture should preferably be performed with urine collected by cystocentesis, followed by catheterisation or free-catch (midstream voiding or manual expression) in to order minimize sample contamination. The use of free-catch urine in companion animals is controversial among authors (Bartges *et al*., 2004; Weese *et al*., 2011, Soerensen *et al*., 2016). The quantitative urine culture is the gold standard for the diagnosis of significant bacteriuria (Bartges *et al*., 2004; Weese *et al*., 2011) because it accounts for the number of colony forming units per urine volume and true bacteriuria is adjusted to the urine collection method used (Bartges *et al*., 2004). Ideally, urine culture should be followed by antimicrobial susceptibility testing (AST) of the isolated bacteria to guide or adjust antimicrobial therapeutics and to gather epidemiological data on local UTI aetiology and susceptibility patterns (Weese *et al*., 2011).

Aetiology

UTIs are usually caused by bacteria and more rarely by fungi and viruses (Forrester *et al*., 1999; Pressler *et al.*, 2003). *Escherichia coli* (uropathogenic *E. coli* - UPEC) is the main bacteria isolated in all types of UTIs, although other Gram-negative and Gram-positive bacteria may also be implicated. The frequency of each bacteria genera varies according to the study likely reflecting different geographical/temporal trends as well as different inclusion criteria. (Bush, 1976; Wooley and Blue, 1976; Forrester *et al*., 1999; Hess *et al*., 2000; Ling *et al.*, 2001; Cohn *et al*., 2003; Pressler *et al.*, 2003; Torres *et al*., 2005; Bailiff *et al*., 2006; Litster *et al.*, 2007; Mayer-Roenne *et al*., 2007; Passmore *et al*., 2008; Martinez-Ruzafa *et al*., 2012; Dorsch *et al.*, 2015; Moyaert *et al*., 2017).

Studies conducted in the Antibiotic Resistant Laboratory showed that *E. coli* is indeed the most frequently isolated bacteria from companion animals with UTI in the Lisbon area (Marques *et al*., 2018b) and corroborated previous suspicions that the UTI aetiology varies significantly between cats and dogs (Marques *et al*., 2016; Marques *et al*., 2018a).

After *E. coli*, cats have high frequency of UTIs caused by *Enterococcus* spp. and *Staphylococcus* spp. (Wooley and Blue, 1976; Bailiff *et al*., 2006; Litster *et al*., 2007; Mayer-Roenne *et al*., 2007; Bailiff *et al*., 2008; Passmore *et al*., 2008; Martinez-Ruzafa *et al*., 2012; Dorsch *et al*., 2015; Marques *et al*., 2016; Moyaert *et al*., 2017; Marques *et al*., 2018a; Teichmann-Knorrn *et al*., 2018). *Enterococcus* spp. are significantly more common in cats than in dogs (Marques *et al*., 2016; Marques *et al*., 2018a). *Enterococcus faecalis* is the most prevalent, followed by *Enterococcus faecium,* which is rarely isolated (Litster *et al*., 2007, Mayer-Roenne *et al*., 2007; Marques *et al*., 2018a). Several *Staphylococcus* species may cause UTI in cats (Litster *et al*., 2007; Marques *et al*., 2018a). Litster *et al*. (2007) highlighted the high frequency of UTIs caused by *Staphylococcus felis* in cats from Australia. The same was observed in cats with UTI from Portugal (Lisbon) (Marques *et al.,* 2018b). Other bacteria causing UTIs in cats include *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Pasteurela spp.*, *Klebsiella pneumoniae*, among others (Wooley and Blue, 1976; Lekcharoensuk *et al*., 2001; Bailiff *et al*., 2006; Bailiff *et al*., 2008; Litster *et al*., 2007; Marques *et al*., 2016; Marques *et al*., 2018a).

P. mirabilis is significantly more common in dogs with UTI, usually being the second most frequent isolated Enterobacteriaceae after *E. coli* (Wooley and Blue, 1976; Ling *et al*., 2001; Cohn *et al.*, 2003; Marques *et al*., 2016; Moyaert *et al.*, 2017; Marques *et al*., 2018a). Other bacterial causes of UTI in dogs also include *Staphylococcus* spp., *Enterococcus* spp. and more rarely *Pseudomonas* spp., *Klebsiella* spp., *Streptococcus* spp. (Forrester *et al.*, 1999; Norris *et al.*, 2000; Ling *et al.*, 2001; Prescott *et al*., 2002; Cohn *et al*., 2003; Marques *et al*., 2016; Marques *et al*., 2018a). Although several *Staphylococcus* species may cause UTI in dogs, *Staphylococcus pseudintermedius* predominates (Prescott *et al*., 2002; Cohn *et al*., 2003; Penna *et al.*, 2010;

Marques *et al*., 2018b). Interestingly, dogs with complicated/recurrent UTIs seem to have higher frequencies of less common bacteria such as *K. pneumoniae*, *Enterococcus* spp. and *Pseudomonas* spp. (Forrester *et al*., 1999; Norris *et al*., 2000; Torres *et al*., 2005). Additionally, some studies have suggested that there are some differences according to the dog gender (Norris *et al*., 2000; Ling *et al*., 2001; Cohn *et al*., 2003).

Antimicrobials for UTI treatment in companion animals

The increasing antimicrobial resistance trends observed over the last decades in human and veterinary medicine are a worldwide concern that requires a One Health approach (World Health Organization [WHO], 2017b). The antimicrobial resistance selective pressure due to antimicrobial use is believed to be one key contributing factor (Guardabassi *et al*., 2005; Pomba *et al*., 2017; World Health Organization [WHO], 2017a). Since antimicrobials are the cornerstone for UTI treatment and are frequently required, correct diagnosis and proper antimicrobial selection is crucial to avoid antimicrobial misuse (Weese *et al*., 2011). The ISCAID guidelines for UTI treatment in companion animals propose a rational list of antimicrobials that should be used according to the type of UTI (Weese *et al*., 2011) (Table 1).

The WHO (2017a) groups the antimicrobials according to their importance to human medicine into three categories: important, highly important and critically important antimicrobials (CIA). Furthermore, CIAs may also be divided into high or highest priority antimicrobials based on 3 additional prioritisation criteria (WHO, 2017a). It should be noted that several antimicrobials approved for UTI treatment in small animal veterinary medicine are also used in human medicine and belong to CIAs of high and highest priority (Table 1). Therefore, the rational use of antimicrobials in small animal veterinary medicine is of the upmost importance.

These ISCAID guidelines are general recommendations that need to be properly adjusted considering the specific geographic antimicrobial resistance rates, antimicrobial availability and prescribing regulations (Weese *et al*., 2011). Given the importance of this subject, the Antibiotic Resistance Laboratory has conducted over the last years important antimicrobial resistance surveillance studies to determine the antimicrobial resistance temporal trends and underling resistance mechanisms of bacteria isolated from companion animals with UTI from Portugal (Lisbon) and Europe (Féria *et al*., 2000; Féria *et al*., 2002; Caniça *et al*., 2004; Pomba *et al*., 2009; Oliveira *et al*., 2014; Pomba *et al*., 2014a; Marques *et al*., 2016; Marques *et al*., 2018a; Marques *et al*., 2018b; Belas *et al*., 2019; Marques *et al*., 2019).

Antimicrobial resistance surveillance in companion animals

The European Antimicrobial Resistance Surveillance Network (EARS-Net) actively gathers and reports annual data on antimicrobial resistance in human invasive bacteria from several European countries (European Centre for Disease Prevention and Control [ECDC],

2017). These EARS-Net reports show remarkable geographical differences in antimicrobial resistance frequencies among European countries as well as increasing trends in resistance to CIAs (ECDC, 2017).

Such important surveillance programs are lacking in small animal veterinary medicine. There have been only few national antimicrobial resistance surveillance networks in place for companion animals in Germany, Sweden and France (Swedres-Svarm, 2016; Moyaert *et al*., 2017). In 2008, the European Animal Health Study Centre started an initiative (Compath) gathering bacterial isolates from companion animals in Europe and just recently published data regarding UTI isolates from 2008-2010 (Moyaert *et al*., 2017). Moyaert *et al*., (2017) reported overall high susceptibility to all tested antimicrobials (e.g. >90% for most antimicrobials in *E. coli*). However, since the antimicrobial resistance frequencies were presented for all countries as a group and temporal trends were not analysed (Moyaert *et al*., 2017), it was not possible to perceive any geographical differences. Overall high antimicrobial susceptibility frequencies were also detected in previously published data from Sweden (2009, 2014) (Swedres-Svarm, 2016; Windahl *et al*., 2014), Norway (2003-2009) (Lund *et al*., 2014) and Switzerland (*E. coli*, 1999-2001) (Lanz *et al*., 2003).

In another European study, Kroemer *et al*. (2014) found lower antimicrobial susceptibility rates among *E. coli* and *P. mirabilis* from companion animals with UTI isolated in 2002-2009. Again, results were presented for all countries as a group (Kroemer *et al*. 2014). Notably, *P. mirabilis* showed trimethoprim-sulfamethoxazol resistance of about ~53% (Kroemer *et al*., 2014). Higher levels of antimicrobial resistance in bacteria from companion animals with UTI have also been reported in Portugal (e.g. *E. coli* 25% to cephalotin, 19% to amoxicillin/clavulanate) (Féria *et al*., 2002), Brazil (e.g. staphylococci 28-74% to all tested antimicrobials, 2006-2007; *E. coli* 40% to aminoglycosides, 40% to sulfonamides, 16% to fluoroquinolones) (Penna *et al*., 2010; Osugui *et al*., 2014), Cornell USA (e.g. *E. coli* ~35% to ampicillin, ~70% to cephalotin, ~20% to enrofloxacin, ~40% to gentamicin) (Cummings *et al*., 2015); Taiwan (*E. coli* 50% amoxicillin, 39% trimethoprim-sulphamethoxazole, 2010-2011) (Chang *et al*., 2015), Australia (e.g. *E. coli* 29% to amoxicillin/clavulanate, 5-9% to ceftriaxone, 2013) (Saputra *et al.,* 2017), Switzerland (*E. coli*, 10-35% to third-generation cephalosporin [ESBL-producers], 2012-2016) (Zogg *et al*., 2018b), Belgium (e.g. *E. coli* 12% to amoxicillin/clavulanate, 17% to enrofloxacin, 2010-2012) (Criel *et al*., 2015), Virginia USA (*E. coli*, 18% to amoxicillin/clavulanate, 15% to trimethoprim/sulphamethoxazole,1986-1996) (Forrester *et al*. 1999) and Italy (2013-3015) (Rampacci *et al*., 2018). Furthermore, changing antimicrobial resistance temporal trends have been reported in bacteria isolated from different companion animal infections (Normand *et al*., 2000a; Authier *et al*., 2006; Thompson *et al*., 2011; Beever *et al*., 2015; Couto *et al*., 2016), including in uropathogenic bacteria from California (fluoroquinolones, 1992-2001) (Cohn *et al*., 2003; Cooke *et al*., 2002), Canada (fluoroquinolones, 1984-1998; several antimicrobial, 2002-

2007) (Prescott *et al*., 2002; Ball *et al*., 2008), United Kingdom (enrofloxacin, cephalexin and oxytetracicline, 1999-2009) (Hall *et al*., 2013) and in New Zealand (amoxicillin clavulanate, cephalotin, enrofloxacin, 2005-2012) (McMeekin *et al*., 2016).

Despite the apparently significant number of studies published regarding the antimicrobial resistance trends in bacteria isolated from companion animals with UTI, the comparison of published data is frequently difficult. Most of the studies that report antimicrobial resistance frequencies use different inclusion criteria (e.g. diabetic animals, recurrent UTI) (Bailiff *et al*., 2006), were conducted at different time periods, group results from different infection sites (Normand *et al*., 2000a; Normand *et al*., 2000b; Authier *et al*., 2006; Pedersen *et al*., 2007; Harada *et al*., 2012; Beever *et al*., 2015), combine different bacteria genera (Ball *et al*., 2008; Hall *et al*., 2013; Dorsch *et al*., 2015; Wong *et al*., 2015; Rampacci *et al*., 2018; Teichmann-Knorrn *et al*., 2018) and group data from several countries (Meunier *et al*., 2004; Kroemer *et al*., 2014; Moyaert *et al*., 2017). Knowledge of the geographic distribution of antimicrobial resistance, as obtained by surveillance networks in human medicine, is essential to identify the countries where efforts should be made to improve awareness and implement new strategies.

In a collaboration with 16 veterinary microbiology laboratories from 14 European countries, the Antibiotic Resistance Laboratory team coordinated a multicentric study to determine the European distribution of resistance in bacteria isolated from companion animals with UTI. This study showed striking geographical differences on *E. coli* and *P. mirabilis* antimicrobial resistance between some Northern (Denmark and Sweden) and Southern (Italy, Greece, Portugal and Spain) European countries (Marques *et al*., 2016). Overall, Southern countries showed higher resistance towards the main antimicrobials used in small animal veterinary medicine, including third generation cephalosporins and fluoroquinolones (Marques *et al*., 2016) (Figure 1). One limitation from this study that could have biased these finding to some extent was the use of different antimicrobial testing methods and interpretation criteria in some European veterinary microbiology laboratories. However, the wide differences in antimicrobial resistance detected between some Northern and Southern countries (e.g. 2.88% and 48.15% amoxicillin/clavulanate resistance in Denmark and Portugal, respectively) are likely a result from true geographic differences (Marques *et al*., 2016). It is interesting to note that the European distribution of *E. coli* resistance from companion animals with UTI resembled that of EARS-Net reports about human invasive isolates (ECDC, 2017). Since the samples from most countries were obtained from a single veterinary microbiology laboratory these findings may not represent the entire country. Nevertheless, these results should prompt the Southern countries to further investigate this issue.

Prior to this European study, there was little updated information about the antimicrobial resistance of bacteria causing UTIs in companion animals from Portugal (Féria *et al*., 2000; Féria *et al*., 2002; Pomba *et al*., 2008). To better understand the antimicrobial resistance temporal

trends in uropathogenic bacteria from companion animals in Portugal (Lisbon), the Antibiotic Resistance Laboratory team conducted a retrospective study over 16 years (Marques *et al*., 2018a). Notably, a significant increase in Enterobacteriaceae antimicrobial resistance to the main antimicrobials used for UTI treatment in small animal veterinary medicine was observed in companion animals from Portugal (Lisbon) (Figure 2). Furthermore, a significant increase in the detection of methicillin resistant *Staphylococcus pseudintermedius* was also detected (Marques *et al*. 2018a).

The selection of antimicrobial resistance is a complex and multifactorial process (Prescott, 2017). Efforts to reduce high antimicrobial resistance frequencies and increasing trends are urgent. The prescription of empirical antimicrobial treatment is sometimes necessary prior to culture to relieve the patient discomfort and prevent systemic infection (Weese *et al*., 2011). As part of the rational use of antimicrobials, the empirical choice of antimicrobials should rely on first-line antimicrobials and then adjusted (escalation/de-escalation) based on antimicrobial susceptibility data if necessary (Weese *et al*., 2011). The choice of the appropriate antimicrobial should always be supported on culture and AST (Weese *et al*., 2011) to avoid the misuse of antimicrobials and therefore contribute to decrease the local antimicrobial selective pressure. In fact, Sørensen *et al*. (2018) has showed that a high percentage of dogs with suspected UTI were unnecessarily treated regardless of the diagnostic work up conducted prior to culture and that second line antimicrobials were frequently miss prescribed. Interestingly, an European study reported that veterinarians, for instance, in Sweden were 15.64 times more likely to conduct a AST to guide antimicrobial choice than in Spain (de Briyne *et al*., 2013). All these factors likely contribute to the increase in antimicrobial resistance and reveal the need for the implementation of antimicrobial stewardship programs in small animal veterinary medicine.

Another interesting finding that points to the overuse of antimicrobials in companion animals with UTI is that the recommended treatment duration in companion animals is significantly longer than in humans (e.g. uncomplicated UTI: 3-5 days in humans, 7-14 days in companion animals) (Weese *et al*., 2011; Smelov *et al*., 2016). It should be noted that the treatment duration currently recommended in companion animals are supported by little scientific evidence (Weese *et al*., 2011). Although some studies have reported short-duration antimicrobial treatment protocols for UTI in companion animals (Westropp *et al*., 2012; Clare *et al*., 2014), studies comparing the same antimicrobial regime with differing durations are lacking (Jessen *et al.*, 2015).

Multidrug resistance bacteria causing UTIs from companion animals

Companion animals with UTI have high bacteria concentration in the urine, thus potentially contributing to its dissemination into the living environment. Therefore, the detection

of multidrug resistant (MDR) bacteria in companion animals with UTI creates important therapeutic limitations and also raises public health concerns (Pomba *et al*., 2017).

MDR bacteria are increasingly being detected in companion animals with UTI and are frequently associated with clinically relevant and mobile resistance mechanisms such as extended spectrum beta-lactamases (ESBLs) and carbapenemases in Enterobacteriaceae and the *mecA* gene in staphylococci (Prescott *et al*., 2002; Pomba *et al*., 2008; Pomba *et al*., 2010; Harada *et al*., 2012; Pomba *et al*., 2013; Osugui *et al*., 2014; Wagner *et al.*, 2014; Windahl *et al*., 2014; Chang *et al*., 2015; Thungrat *et al.*, 2015; Wong *et al*., 2015; Marques *et al.* 2018a; Zogg *et al*., 2018b; Belas *et al*., 2019; Marques *et al.* 2019).

In the 16-yearlong retrospective study conducted in Portugal (Lisbon) at the Antibiotic Resistance Laboratory, a significant increase in the detection of MDR Enterobacteriaceae from companion animals with UTI was detected (Marques *et al.* 2018a). Furthermore, in the study conducted in collaboration with the European veterinary microbiology laboratories, Portugal (Lisbon) and the Southern countries were, again, among the geographic locations with higher frequency of MDR *E. coli* and *P. mirabilis* (Marques *et al*., 2016).

The increase in the detection of MDR *E. coli* and *P. mirabilis* in companion animals from Portugal (Lisbon) was strongly associated with the dissemination of ESBL and AmpC beta-lactamases (Marques *et al.* 2018a). The production of beta-lactamases is the most common beta-lactam resistance mechanism in clinically relevant Gram-negative bacteria (Bush and Jacoby, 2010). Beta-lactamases are frequently plasmid mediated leading to its rapid worldwide dissemination through horizontal transfer and dissemination of high-risk clones (Cantón and Coque, 2006; Fernandes *et al*., 2013). Furthermore, plasmids frequently encode for antimicrobial resistance to different classes of antimicrobials, thus contributing to the dissemination of MDR phenotypes (Cantón and Coque, 2006).

The Antibiotic Resistance Laboratory has contributed significantly to the knowledge of the epidemiology of beta-lactamase enzymes in Gram-negative bacteria isolated from companion animals with UTI, including ESBLs and carbapenemases (Féria *et al*., 2002; Caniça *et al*., 2004; Pomba *et al*., 2008; Pomba *et al*., 2013; Marques *et al*., 2018a; Marques *et al*., 2018b; Belas *et al*., 2019; Marques *et al*., 2019).

The spread of third-generation cephalosporin resistant Enterobacteriaceae has recently been considered by the WHO as a Priority 1 concern (WHO, 2017b). The CTX-M family is endemic worldwide and has become the most frequent ESBL in bacteria causing health-care and community associated infections in humans (Cantón and Coque, 2006; Fernandes *et al*., 2013; Doi *et al*., 2017). There is host and geographic variation in the distribution of CTX-M enzymes; nevertheless, some enzymes, such as CTX-M-15, seem to be disseminated worldwide in humans and companion animals (Cantón and Coque, 2006; Coque *et al*., 2008; Nicolas-

Chanoine *et al*., 2008; Pomba *et al*., 2008; Smet *et al*., 2010; Ewers *et al*., 2012; Bevan *et al*., 2017; Marques *et al*., 2018a; Marques *et al*., 2019).

There is a strong association between the *E. coli* O25b:H4-B2-ST131 clonal lineage and the dissemination of CTX-M-15 ESBL (Coque *et al*., 2008; Nicolas-Chanoine *et al*., 2008; Doi *et al*., 2017). This clonal lineage is also relevant because it exhibits a large virulence gene profile and is an important uropathogen in humans (Nicolas-Chanoine *et al*., 2008; Vimont *et al*., 2012). This important CTX-M-15-producing *E. coli* clonal lineage (O25b:H4-B2-ST131) has sporadically been reported in companion animals, including from Portugal (Nicolas-Chanoine *et al*., 2008; Pomba *et al*., 2009; Ewers *et al*., 2010; Pomba *et al*., 2013; Belas *et al*. 2019). Just recently, some *E. coli* isolated from companion animals with UTI in Portugal were found to belong to the fluoroquinolone resistant CTX-M-15-producing O25b:H4 B2-ST131-H30Rx subclone, representing its first description in companion animals living in Europe. Moreover, these subclones belonged to the virotype D, which confirms their pathogenicity and virulent characteristics (Belas *et al*., 2019).

Although *K. pneumoniae* is less frequent in companion animals with UTI (Marques *et al*., 2016; Marques *et al*., 2018), it is still a major pathogen that is increasingly associated with the dissemination of ESBLs and carbapenemases (Navon-Venezia *et al*., 2017; Ewers *et al*., 2014; Stolle *et al*., 2013; Schmiedel *et al*., 2014; González-Torralba *et al*., 2016). Several studies have shown that the ST15-CTX-M-15 clonal lineage predominates in companion animal infections by third-generation cephalosporin *K. pneumoniae* (Ewers *et al*., 2014; Maeyama *et al*., 2018; Marques *et al*., 2019). A high frequency of MDR *K. pneumoniae* ST15-CTX-M-15 was also detected in companion animals with UTI from Portugal (Lisbon) (Marques *et al*., 2019). Furthermore, other MDR high-risk clonal lineages disseminated in Portuguese Hospitals (Manageiro *et al*., 2015), such as the ST11 and ST147, were also detected (Marques *et al*., 2019).

The AmpC cefalosporinases are still regarded as less frequent than ESBLs, with CMY-2 being the most disseminated in humans and companion animals (Smet *et al*., 2010; Ewers *et al*., 2012). Interestingly, the studies conducted in Portugal (Lisbon) showed a significant increase in the detection of CMY-2 producing *E. coli* ST648 and *P. mirabilis* in companion animals with UTI (Marques *et al*., 2018a; Marques *et al*., 2018b). This CMY-2 increase is worrisome because these enzymes show stronger β-lactamase activity than ESBLS (Jacoby, 2009) and may exhibit resistance to carbapenems due to the presence of other resistance mechanisms (e.g. porin deficiency) (Chia *et al*., 2009). Furthermore, all *E. coli* and *P. mirabilis* from this study (Marques *et al*., 2018a; Marques *et al*., 2018b) were MDR which creates great therapeutic limitations and highlights their clinical relevance.

The choice of an appropriate antimicrobial for the treatment of infections caused by MDR bacteria is a true challenge in small animal veterinary medicine. Not rarely, the lack of therapeutic

options require the use of off-label of antimicrobials as demonstrated by Pomba *et al*. (2010). Unlike AmpC and carbapenemases, ESBL-producing Enterobacteriaceae may be susceptible in vitro to amoxicillin/clavulanic acid (Paterson and Bonomo, 2005). Due to limited research data, beta-lactam/beta-lactam inhibitor combinations are not considered as suitable first line options for the treatment of serious infections caused by ESBL-producing bacteria (Paterson and Bonomo, 2005). However, the successful treatment of UTIs caused by some fully amoxicillin/clavulanate susceptible ESBL-producing *E. coli* in humans has been reported. Presumably, the high concentration of amoxicillin/clavulanate achieved in urine are responsible for such success (Lagacé-Wiens *et al*., 2006; Beytur *et al*., 2015). In a Pilot study conducted in a cat with a MDR ST15-CTX-M-15 producing *K. pneumoniae* UTI infection, the use of amoxicillin/clavulanate was tested (Marques *et al*., 2017). Although a definite cure was not achieved, the significant decrease in bacteriuria detected was a promising finding. Additional studies are now being conducted in the Antibiotic Resistance Laboratory to fully evaluated this therapeutic approach in small animal veterinary medicine.

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Table 1: Antimicrobials used for UTI treatment in dogs and cats.

Antimicrobials	WHO (2017a) classification[1]	Companion animals[2]
Beta-lactams		
Amoxicillin	CIA - HP	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI.
Amoxicillin/clavulanic	CIA - HP	Unknown advantage over amoxicillin alone. If amoxicillin resistance rates are high locally, may be a good first-line option for UTI treatment and empirical treatment of uncomplicated and complicated UTI.
Second -generation cefalosporins	HIA	Second-line option for UTI treatment.
Third-generation cefalosporins	CIA – HestP	Second-line option for UTI treatment.
Carbapenems	CIA - HP	Last-resort antimicrobial. Prescribed off-label.
Aminoglycosides	CIA - HP	Not recommended for routine use due to side effects. Although not included in Weese *et al*. (2011) guidelines, gentamicin has been shown to be useful for UTI treatment (Ling and Ruby, 1979).
Chloramphenicol	HIA	Off-label use. Recommended for multidrug resistant bacteria.
Doxycycline	HIA	Not recommended for routine use; nevertheless, its usefulness in UTI treatment has been demonstrated (Wilson *et al*., 2006).
Fluoroquinolones	CIA - HestP	Second-line option for UTI treatment. Considered a good first-line option for empiric antimicrobial treatment of pyelonephritis.
Nitrofurantoin	IA	Second-line antimicrobial. Off-label use. Reserved for uncomplicated UTIs caused by multidrug resistant bacteria.
Trimethoprim/sulfamethoxazole	HIA	First-line option for UTI treatment. First-line option for empirical treatment of uncomplicated and complicated UTI.

Legend: CIA, critically important antimicrobial; HP, high priority antimicrobial; IA, Important antimicrobial; HP, high priority; HestP, Highest priority;[1] as defined by WHO (2017a); [2]Major data according to Weese *et al*. (2011).

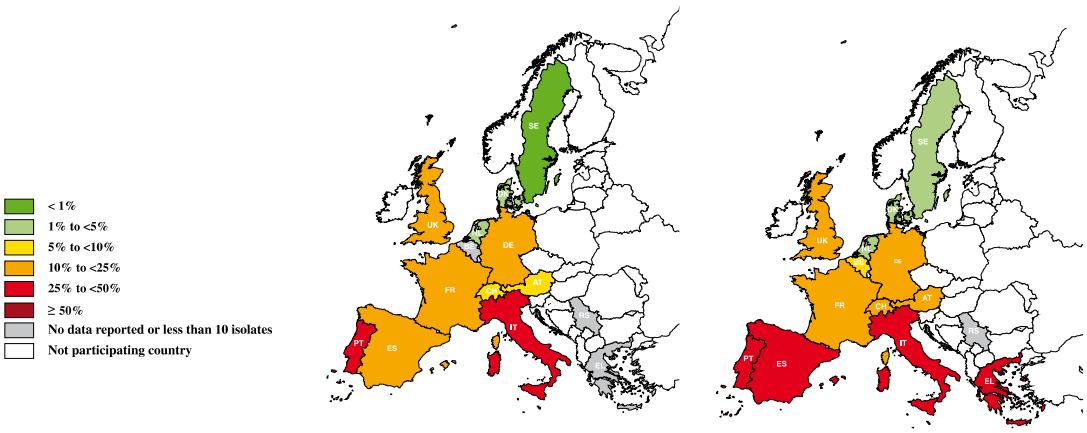

Figure 1: *Escherichia coli* resistance to third-generation cephalosporin (left) and fluoroquinolone (right) in companion animals with UTI (adapted from Marques *et al*., 2016).

Legend: *E. coli* antimicrobial resistance by country in the years 2012–2013. Countries: AT- Austria; BE- Belgium; DK- Denmark; FR- France; DE- Germany; EL- Greece; IT- Italy; NL- the Netherlands; PT- Portugal; RS- Serbia; ES- Spain; SE- Sweden; CH- Switzerland; UK- United Kingdom.

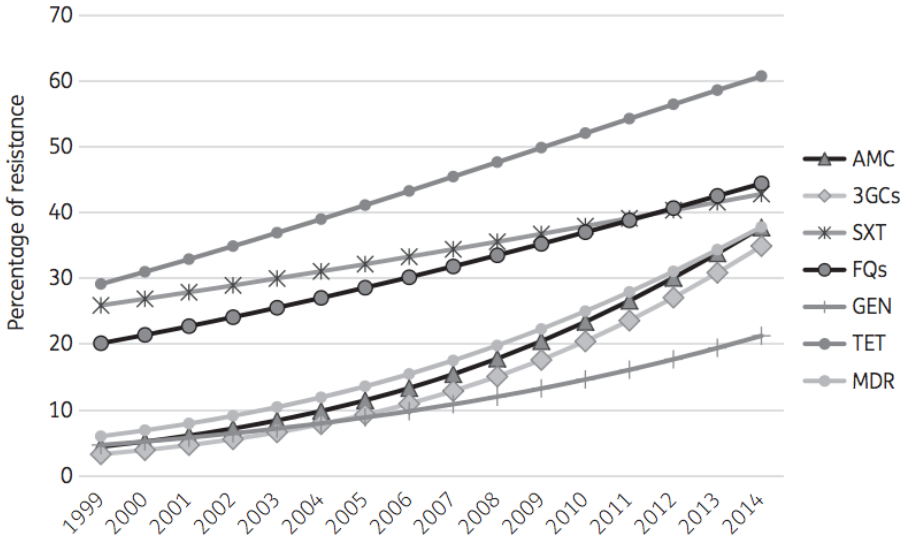

Figure 2: Trends in antimicrobial resistance in Enterobacteriaceae causing UTI in companion animals from Portugal (Lisbon), 1999-2014 (adapted from Marques *et al*., 2018b).

Legend: AMC, amoxicillin/clavulanate; 3GCs, third-generation cephalosporins, SXT, trimethoprim-sulfamethoxazole; FQs, fluoroquinolones; GEN, gentamicin; TET, tetracycline; MDR, multidrug-resistant.

Annex 2 - The public health risk of companion animal to human transmission of antimicrobial resistance during different types of animal infection.

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The public health risk of companion animal to human transmission of antimicrobial resistance during different types of animal infection

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Abstract

Antimicrobial resistance represents a major threat to human health. As a result, we are faced with potential antimicrobial therapeutic failure, thus forcing physicians to use last resort antimicrobials, such as carbapenems, glycopeptides or polypeptides. During the last fifty years, the number of companion animals has substantially increased and there is a growing concern related to the use of antimicrobials in companion animals as a potential source for antimicrobial resistance to humans. Problems related with antimicrobial resistance and infection control in small animal hospitals are mimicking those in human hospitals. Transmission of pathogens or resistance genes such as methicillin-resistant staphylococci, extended spectrum beta-lactamase- or carbapenemase-producing and colistin-resistant Enterobacteriaceae between people and their pets have been documented or suggested. The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals were recently reviewed by the European Medicine Agency which warned to the existence of antimicrobial resistance microbiological hazards coming from companion animals to humans. The magnitude to which these occur, and the risks posed by the different animal species is still inadequately studied. This is the main goal of the JPI-EC-AMR JTC 2016 Pet-Risk Consortium (Portugal, Germany, Switzerland, UK, and Canada) JPIAMR/0002/2016 under the CIISA Antibiotic Lab Team Leader Coordination.

Keywords: Antimicrobial resistance, animal infection, public health risk.

The antimicrobial use in veterinary medicine

The increase in antimicrobial resistance represents a major threat to human and animal health (WHO, 2017a). As a result, we are now faced with the reduction of treatment options and with potential therapeutic failure leading veterinarians to use antimicrobials off-label and physicians to use last resort antimicrobials.

There is a growing concern related to the use of antimicrobials in food-producing and companion animals as a potential source for antimicrobial resistance to humans (Greko *et al*., 2009; Catry *et al*., 2010; van Duijkeren *et al*., 2014; Pomba *et al*., 2017). In fact, it is known that the use of antimicrobials increases the risk of antimicrobial resistance and the risk of colonization with antimicrobial-resistant bacteria (Barza and Travers, 2002; Belas *et al*., 2014).

Since 2010, the European Medicine Agency started reporting data on antimicrobial sales for companion animals (EMA, 2017b). Beta-lactams, including potentiated penicillins, were the most frequently sold for companion animals in most European countries, including Portugal (EMA, 2017b). Furthermore, fluoroquinolones were the second most sold in Portugal.

It should be noted that the most sold antimicrobials in companion animals worldwide overlap those routinely used in human medicine and are considered as critically important antimicrobials to humans by the World Health Organization (WHO) (WHO, 2017b). Interestingly, the problems of antimicrobial resistance development and infection control in small animal hospitals are mimicking those in human hospitals (ECDC, 2009). Furthermore, in contrast to food-producing animals, the prescription of antimicrobials only approved for human use may occur under the cascade principles in companion animals (Pomba *et al*., 2017). This represents an additional antimicrobial resistance selective pressure towards last resorts antimicrobials and warrants the need for a One Health approach to fighting the dissemination of antimicrobial resistance.

Risk of transfer of antimicrobial-resistant bacteria

The number of companion animals has significantly increased over the last 50 years (Guardabassi *et al*., 2004; Pomba *et al*., 2017). The closer contact between owners and companion animals creates opportunities for pathogen interchange through direct and indirect contact (Guardabassi *et al*., 2004; Damborg *et al*., 2016).

The public health risks associated with the transfer of antimicrobial-resistant bacteria from companion animals have been reviewed in the European Medicine Agency and its Antimicrobial Working Party reflection paper (Pomba *et al*., 2017). Pomba *et al*. (2017) alerted for existence of several antimicrobial resistance microbiological hazards coming from companion animals to humans (Table 1).

The concerns surrounding the role of companion animals in the dissemination of resistant bacteria to humans are strengthened by numerous studies reporting the colonization and/or infection of companion animals with bacteria harboring clinically relevant antimicrobial resistance

mechanisms or bacteria belonging to high-risk clonal lineages to humans (Guardabassi *et al*., 2004; Damborg *et al*., 2016; Pomba *et al*., 2017).

This has been one of the main focus of the research conducted in the Antibiotic Resistance Laboratory in Enterobacteriaceae, non-Enterobacteriaceae, Staphylococci and Enterococci (Féria *et al*., 2001; Féria *et al*., 2001b; Caniça *et al*., 2004; Delgado *et al*., 2007; Braga *et al*., 2011; Couto *et al*., 2011; Pinho *et al*., 2013; Catry *et al*., 2015; Couto *et al*., 2015; Razuaskas *et al*., 2015a; Razuaskas *et al*., 2015b; Couto *et al*., 2016a; Couto *et al*., 2016b; Razuaskas *et al*., 2016; Pomba *et al*., 2017;; Costa *et al*., 2018; Marconi *et al*., 2018; Marques *et al*., 2018a; Rodrigues *et al*., 2018; Belas *et al*., 2019; Marques *et al*., 2019a; Marques *et al*., 2019b).

Antimicrobial resistance mechanisms and bacteria of concern

Staphylococci

Portugal is among the European countries with higher frequency of methicillin-resistance in invasive *Staphylococcus aureus* from humans (ECDC, 2017). Methicillin-resistant Staphylococcus aureus (MRSA) have been detected in a wide number of animal species (Catry *et al*., 2010; Pomba *et al.*, 2017), including in Portugal (Coelho *et al*., 2011; Couto *et al*., 2014; Beça *et al*., 2015; Couto *et al*., 2015; Couto *et al*., 2016a; Rodrigues *et al*., 2018).

In companion animals, MRSA has been isolated from skin and soft-tissue infections, post-surgical wound infections, urinary tract infections and pneumonia (Catry *et al.*, 2010; Pomba *et al*., 2017). In a study from Portugal, conducted in the Antibiotic Resistance Laboratory, several MRSA were detected in companion animals with skin and urinary tract infections (Couto *et al*., 2016). Notably the MRSA strains isolated from companion animals belonged to CC5 which is a lineage associated with human infection in Portugal (Couto *et al*., 2015; Couto *et al*., 2016).

In fact, the similarity of MRSA clonal lineages isolated from companion animals and humans has been reported worldwide (Weese and Duijkeren, 2010; Pomba *et al*., 2017). In another study about the clonal diversity, virulence patterns and antimicrobial and biocide susceptibility among human, animal and environmental MRSA in Portugal, *S. aureus* clonal lineages from companion animals (CC5 and CC22) were associated with specific sets of virulence genes and often with a lower number of resistance genes than isolates belonging to the livestock associated CC398 (Couto *et al*., 2015). Colonization of companion animals with MRSA has been previously reported ranging from 0% to 7% (Leornardo and Markey, 2008; Catry *et al*., 2010; Pomba *et al*., 2017). In one study from Portugal, 1.4% of cats and 0.7% of dogs were reported to be colonized by MRSA (Couto *et al*., 2014).

The risk of transmission of MRSA between companion animals and humans has been demonstrated highlighting the role of both species in this issue (Damborg *et al*., 2016; Pomba *et al*., 2017). Interestingly, veterinary staff seems to be at higher risk of being colonized by MRSA

(Baptiste *et al*., 2005; Loeffler *et al*., 2005; Catry *et al*., 2010, Pomba *et al*., 2017). Besides MRSA, companion animal health care providers from Portugal also had a high frequency of colonization by methicillin resistant *Staphylococcus epidermidis* (MRSE) (Rodrigues *et al*., 2018). MRSAs colonizing humans from this study belonged to the major human healthcare clone in Portugal (ST22-t032-IV), the livestock-associated MRSA (ST398-t108-V) and to the New York-/Japan-related clone (ST105-t002-II) (Rodrigues *et al*., 2018). Furthermore, *S. epidermidi*s is an important nosocomial pathogen responsible for life-threatening infections associated with the use of medical devices and in immunocompromised individuals, whose management is hindered by frequent resistance to antimicrobials (Costa *et al*., 2018).

Most infections in companion animals are caused by *Staphylococcus pseudintermedius*, especially in dogs (Couto *et al*., 2016a; Couto *et al*., 2016b). The detection of multidrug-resistant methicillin-resistant *S. pseudintermedius* (MRSP) is increasingly being reported leading to significant therapeutic limitation in small animal veterinary medicine (Couto *et al*., 2014b; Couto *et al*., 2016a; Couto *et al.,* 2016b; Pomba *et al*., 2017). A significant increase in the detection of multidrug-resistant MRSP has been recently noted in companion animals from Portugal (Lisbon) (Couto *et al*., 2016a). Although methicillin-susceptible *S. pseudintermedius* isolates are genetically diverse, a limited number of MRSP clones have spread worldwide resembling the worldwide dissemination of MRSA (Van Duijkeren *et al*., 2011; Pomba *et al.*, 2017). Like MRSA, the emergence of MRSP represents a great problem for small animal veterinary medicine since *S. pseudintermedius* is the primary staphylococcal species colonizing healthy dogs and cats. MRSP colonization is more common in dogs than in cats. Furthermore, MRSP can cause many types of infections in companion animals as skin and ear infections, surgical site infections, gingivitis, hepatitis, urinary tract infections, respiratory infections, arthritis, peritonitis and septicaemia (Van Duijkeren *et al*., 2011; Pomba *et al*., 2017). It is important to keep in mind that veterinary hospitals and clinics play an important role in the dissemination control of MRSP (Pomba *et al*., 2017).

In Portugal, the Antibiotic Resistance Laboratory has conducted extensive studies about the *S. pseudintermedius* (MRSP and MSSP) colonization and infection in dogs and cats to characterize their clonality, antimicrobial susceptibility, biocide susceptibility and immunogenic properties (Couto *et al*., 2014; Couto *et al*., 2015b; Couto *et al*., 2016; Couto *et al*., 2016b). A worrying finding from these studies was the significant increase in staphylococci resistance, mainly *S. pseudintermedius,* to a large number of antimicrobials over the last 16 years (Couto *et al*., 2016). Importantly, this included an increase in the detection of multidrug-resistant MRSP and the *mecA* gene (Couto *et al*., 2016). The increase of MRSP in Portugal was linked to the dissemination of the S. pseudintermedius clonal lineage ST71-II-III, which is also the most disseminated clonal lineage in dogs and cats from Europe (Kadlec *et al*., 2010; Perreten *et al*., 2010).

Colonization of humans with *S. pseudintermedius* seems to be uncommon and transient, however owners and veterinarians in contact with infected companion animals may have a higher risk of being MRSP positive (Pomba *et al*., 2017). There are some reports of colonization of veterinarians by MRSP that could suggest an occupational risk (Sasaki *et al*., 2007; Ishihara *et al*., 2010; Paul *et al.,* 2011; Soedarmanto *et al*., 2011; Gómez-Sanz *et al*., 2013; Chanchaithong *et al*., 2014; Pomba *et al.,* 2017). Furthermore, in 2014 a cluster of infections in a tertiary hospital due to MRSP clone ST71 was described in humans (Starlander *et al*., 2014).

While MRSA strains isolated from companion animals are mainly related to different human-associated MRSA clones, the scenario for MRSP is different. Diverse SCC*mec* elements occur among the different MRSP genetic lineages, suggesting that the *mec*A gene has been acquired by different *S. pseudintermedius* strains on multiple occasions. (Pomba *et al*, 2017). Transfer of SCC*mec* elements between different staphylococcal species is possible, which is a concern.

Enterococci

Enterococci are opportunistic pathogens that have become an important cause of nosocomial and community-acquired infections, such as septicemia, endocarditis, UTI and diarrhea. Moreover, these bacteria are an important key indicator for several human and veterinary resistance surveillance systems (Torres *et al*., 2018). *Enterococcus faecalis* and *Enterococcus faecium* are the most common species isolated from human and companion animal infections. Enterococci are intrinsically resistant to several antimicrobials which have important therapeutic implications (Torres *et al.* 2018). Therefore, acquired resistance to ampicillin/penicillin and to high-level gentamicin, a classic therapeutic synergetic combination, strongly limits the treatment options against enterococcal infections (Chow, 2000). Such resistance mechanisms have been described in enterococci isolated from companion animals from Portugal (Delgado *et al*., 2007; Marques *et al*., 2019a).

Some studies provide that healthy livestock, wildlife, food-producing animals and companion animals can harbour pathogenic Enterococci that can be transferred via food chain or through close contact with humans. Furthermore, some Enterococci species are able to evolve from being simple commensal bacteria to being pathogenic to humans and animals through the acquisition of virulence factors encoded in mobile genetic elements (Bortolaia and Guardabassi, 2015; Pillay *et al*., 2018).

For instance, the *Enterococcus faecalis* ST16 clonal lineage is considered a zoonotic pathogen and food and industries seem to have contributed to its dissemination (Torres *et al*., 2018). Furthermore, this clonal lineage is frequent among high-level gentamicin resistant strains harboring the bifunctional enzyme (Ruiz-Garbajosa *et al*., 2006). Other important Enterococci high-risk clonal complexes (CC) associated with nosocomial infections in humans include the *E.*

faecalis CC6 (formerly CC2) and the ampicillin-resistant *E. faecium* CC17 (Leavis *et al*., 2006; Kuch *et al*., 2012).

Due to its clinical relevance, the Antibiotic Resistance Laboratory has contributed with epidemiological studies about the antimicrobial resistance and population structure of enterococci isolated in Portugal (Delgado *et al*. 2007; Pomba *et al*., 2010; Braga *et al*., 2011; Braga *et al.*, 2013; Marques *et al*., 2019a). In one of these studies, the first report of a biocide resistance mechanism in *E. faecalis* and its dissemination amongst the genus Enterococcus was reported (Braga *et al*., 2011).

Ampicillin-resistance and/or high-level gentamicin resistance in enterococci from companion animals with UTI in Portugal (Lisbon) over 16 years was low when compared with the resistance frequencies detected in Enterobacteriaceae (Marques *et al*., 2019a). However, many of these isolates belonged to *E. faecalis* ST16, *E. faecalis* CC6 and to the ampicillin-resistant *E. faecium* CC17. Interestingly, a previous study has shown that healthy dogs seem to be reservoirs of ampicillin-resistant *E. faecium* CC17 (Damborg *et al*., 2009).

The acquired resistance to vancomycin due to *van* gene carriage is another resistance mechanism of great importance in human medicine (Pomba *et al*., 2017). Ampicillin-resistance in *E. faecium* from Europe seams to often predict the increase in the rates of vancomycin-resistant enterococci (VRE) within some years (Werner *et al*., 2008). Although, the level of ampicillin-resistant *E. faecium* in companion animals with UTI was low, higher frequencies have been reported in other parts of Europe (Damborg *et al*., 2009). Therefore, active surveillance is imperative.

Healthy dogs and cats may become colonized by VRE. Furthermore, VRE isolated from companion animals may also belong to clonal lineages associated with hospital-acquired infections (Pomba *et al*., 2017).

Enterobacteriaceae and non-Enterobacteriaceae

There are a large number of studies reporting the detection of extended spectrum beta-lactamases (ESBLs) -producing bacteria in companion animal infections and in colonized animals (Ewers *et al*., 2012; Belas *et al*., 2014; Pomba *et al*., 2014a; Damborg *et al*., 2015; Pomba *et al*., 2017).

Detection of carbapenemase-producing Enterobacteriaceae and non-Enterobacteriaceae are still a rare event; however, reports in healthy and sick animals are increasing by the day, and will likely become a serious problem in the future (Pomba *et al*., 2014b; Chanchaithong *et al*., 2018; Gentilini *et al*., 2018; Grönthal *et al.*, 2018; Köck *et al*., 2018).

The Antibiotic Resistance Laboratory has made the first description of an OXA-23-producing ST2 MDR *Acinetobacter baumannii* in a cat with urinary tract infection (UTI) (Pomba *et al*., 2014a). Just recently, the transmission of a canine clinical NDM-5 *Escherichia coli* between

an infected dogs and humans was confirmed for the first time (Grönthal *et al*., 2018) giving additional scientific support to the concerns surrounding the close contact of companion animals with humans (Pomba *et al*., 2017).

Although less studied than other Gram-Negative bacteria, *Pseudomonas aeruginosa* is an important pathogen causing otitis and pyoderma in companion animals (Pomba *et al*., 2017). Notably, carbapenem-producing strains have already been detected in dogs (Hyun *et al*., 2018). Also, some infections caused by this bacteria are in association with other pathogens, such as MRSP (Lupo *et al*., 2018).

In an ongoing study conducted in the Antibiotic Resistance laboratory, *P. aeruginosa* causing external otitis in companion animals from Portugal (Lisbon) showed high resistance levels towards fluoroquinolones and aminoglycosides, which are frequently used topically. Furthermore, resistance to imipenem and doripenem was also noted (Marconi *et al*., 2018).

Companion animals have been found to be colonized by *E. coli* and *Klebsiella. pneumoniae* belonging to important clonal lineages to humans (Pomba *et al*., 2014b; Johnson *et al*., 2016; Pomba *et al*., 2017; Marques *et al*., 2018a;Belas *et al*., 2019; Marques *et al*., 2019a). Since many pathogenic bacteria, are thought to make part of the normal gut flora (Podschun and Ullmann, 1998; Drzewiecka, 2016; Johnson *et al*., 2016; Martin *et al*., 2016), gut colonization of companion animals may also represent an important hazard. Interestingly, pet ownership (dogs, cats and other companion animals) was suggested to be a risk factor for human gut colonization by ESBL-producing *E. coli* (Meyer *et al*., 2012).

Regarding Enterobacteriaceae, companion animals from the same household may be colonized and share the uropathogenic *E. coli* O25B: H4: B2-ST131 clonal lineage (Johnson *et al*., 2009; Johnson *et al*., 2016). More importantly, humans and dogs with UTI have been shown to share the index uropathogenic *E. coli* with household members including the family dogs and cats (Murray *et al*., 2004; Johnson and Clabots, 2006). Just recently, in a study conducted by the Antibiotic Resistance Laboratory, companion animals and humans living in close contact were screened for colonization by *K. pneumoniae* and *Proteus mirabilis* (Marques *et al*., 2018a; Marques *et al*., 2019a). Interestingly, some dogs and humans were shown to be colonized in the gut by undistinguishable (by PFGE and MLST) *K. pneumoniae* strains, suggesting the possibility of transmission between dogs and humans (Marques *et al*., 2018a).

Besides beta-lactams, the dissemination of colistin resistance plasmids mcr-1 to 7 has been recently on the spotlight (Yang *et al*., 2018). The dissemination of MDR carbapenemase-producing bacteria in human medicine has led to the need to return to old antimicrobials such as colistin. The recent identification of the colistin resistance gene mcr-1 in food-production animals and companion animals in multiple countries is a concern (Liu *et al*., 2016; Perreten *et al*., 2016; Schwarz *et al*., 2016). In Portugal, mcr-producing Enterobacteriaceae have been identified in retail meat (Figueiredo *et al*., 2016); clinical strains (Campos *et al*., 2016; Mendes *et al*., 2018)

and food producing animals (Kieffer *et al*., 2017; Freitas-Silva *et al*., 2018). Moreover, a recent report of a mcr-1-containing *E. coli* in a person and in multiple dogs and cats heightens these concerns (Zhang *et al*., 2016).

The future

The current scientific knowledge seems to support the suspicion that companion animals may act in the dissemination of resistant and pathogenic bacterial clones to humans and vice versa.

However, several questions still remain answered. Skin (including ear) and urinary tract infections are the most frequent infection in companion animals. Previously published data, including from the Antibiotic Resistance Laboratory (Féria *et al*., 2001; Féria *et al*., 2001b; Caniça *et al*., 2004; Delgado *et al*., 2007; Braga *et al*., 2011; Couto *et al*., 2011; Pinho *et al*., 2013; Catry *et al*., 2015; Couto *et al*., 2015; Razuaskas *et al*., 2015a; Razuaskas *et al*., 2015b; Couto *et a*l. 2016a; Couto *et al.,* 2016b; Razuaskas *et al*., 2016; Pomba *et al*., 2017; Costa *et al*., 2018; Marques *et al*., 2018a;; Rodrigues *et al*., 2018; Belas *et al*., 2019; Marques *et al.*, 2019a; Marques *et al*., 2019b), have shown that bacteria causing skin infections and UTIs in companion animals are sometimes associated with major resistance mechanisms and bacterial clonal lineages. Furthermore, several studies support the sharing/transmission of important bacterial clonal lineages between companion animals and humans (Johnson and Clabots, 2006; Murray *et al*., 2004; Johnson *et al*., 2009; Johnson *et al*., 2016; Marques *et al*., 2018a; Marques *et al*., 2019b). However, the extent to which such transfer occur is still poorly studied. The main goal of the JPI-EC-AMR JTC 2016 Pet-Risk Consortium (Portugal, Germany, Switzerland, UK, Canada) JPIAMR/0002/2016 under CIISA Antibiotic Lab Team Leader Coordination is to clarify the extent of transmission and whether different types of infections may convey additional risk to humans or vice-versa. This project will stand on edge using Next Generation Sequencing technologies to unequivocally evaluate the transmission of clinically relevant antimicrobial mechanisms and pathogenic bacteria.

As a laboratory devoted to the study of antimicrobial resistance in veterinary medicine, it is its mission to reach out to the society (clinicians and owners) in the pursuit of better antimicrobial use practices. The development of antimicrobial stewardship programs as long started in human medicine and are urgently needed in veterinary medicine (Loyd and Page, 2018). Antimicrobial stewardship programs are complex and require the interaction of multidisciplinary teams. Such programs aim at creating strategies to promote the rational use of antimicrobials, improve infection control measures and consequently decrease the spread of pathogenic and resistant bacteria (Loyd and Page, 2018).

Evidence based learning is the key to fight antimicrobial resistance in a One health approach and pursuing the 5Rs of antimicrobial stewardship: Responsibility, Reduction,

Refinement, Replacement and Review (Weese *et al*., 2013; Loyd and Page, 2018). Recently, the European Society of Clinical Microbiology and Infection Diseases Study Group on Veterinary Microbiology started regular post-graduate courses of antimicrobial stewardship in veterinary medicine representing a landmark towards a better future and in which the Antibiotic Resistance Laboratory team leader collaborates as a regular speaker.

The future of antibiotic resistance and pathogenic bacteria is still uncertain, but the Antibiotic Resistance laboratory will continue to focus its efforts in obtaining useful epidemiological data, guide antimicrobial use through the establishment of antimicrobial stewardship programs, and reaching the society to increase awareness and help to improve this worldwide problem.

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Table 1: Microbiological hazards from companion animals to humans identified by EMA (adapted from Pomba *et al*., 2017).

Antimicrobial-resistant bacteria	Type of Hazard	Source
MRSA	direct hazard[1]	dogs, cats and horses
MRSP	direct hazard	dogs, cats and horses
VRE	indirect hazard[2]	dogs and horses
ESBL-producing Enterobacteriaceae	indirect hazard	dogs, cats and horses
Carbapenem-resistant Gram-negative bacteria	indirect hazard[2]	Dogs and cats
Colistin-resistant *E. coli*	indirect hazard	Dogs and cats

Legend: [1]Low number of cases of human infections originating from companion animals.
[2]No human infections originating from companion animals have been reported. However, regarding carbapenems, co-colonization has been recent.